ENZYMATIC AND IMMUNOLOGICAL COMPARISON OF *MYCOBACTERIUM TUBERCULOSIS* AND A CLINICAL ISOLATE OF STREPTOMYCES

by

Zaenab Aljassim

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Thesis Examiners/Examinateurs de thèse:

Dr. Mazen Saleh
(Supervisor/Directeur de thèse)

Dr. Kabwe Nkongolo
(Committee member/Membre du comité)

Dr. Robert Lafrenie
(Committee member/Membre du comité)

Dr. Takuji Tanaka
(External Examiner/Examinateur externe)

Approved for the Faculty of Graduate Studies
Approuvé pour la Faculté des études supérieures
Dr. David Lesbarrères
M. David Lesbarrères
Acting Dean, Faculty of Graduate Studies
Doyen Intérimaire, Faculté des études supérieures

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Abstract

Tuberculosis is a bacterial infection that affects one-third of the global population. The pathogen responsible for the vast majority of these cases is *Mycobacterium tuberculosis* and the current vaccines are insufficiently effective. The current vaccine against Tuberculosis is the live bacille Calmette-Guérin (BCG) vaccine with efficacy varying between 0% and 60% depending on the population demographics. DNA, cellular fractions of the pathogen, and subunit vaccines failed to provide protection beyond what the BCG vaccine can provide. Streptomyces, phylogenetic relatives of the mycobacteria, have been suggested as heterologous systems to formulate new vaccines against Tuberculosis. The main research objective of this study is to establish a functional relationship between *M. tuberculosis* and a clinical isolate of streptomyces using enzymatic and immunological profiling. This clinical isolate was confirmed to be related to *Streptomyces albus*. Enzymatic profiling of the culture filtrate showed that out of a total of 19 enzyme activities investigated, eight were common between *S. albus* and *M. tuberculosis*. These were: alkaline phosphatase, esterase lipases (C8), lipase (C14), leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, and β-glucosidase. Highest levels of acid phosphatase activity was found in the culture filtrate protein (CFP) fraction of *S. albus* cultured in media containing glycine as a nitrogen source but was highest in the cytoplasmic fractions of cells grown with nitrate as a nitrogen source. The opposite was true for alkaline phosphatase where the highest activity was detected in the media with asparagine as a nitrogen source. Alanine dehydrogenase, alcohol dehydrogenase, and catalase/peroxidase showed highest levels in the CFP fraction of the media supplemented with nitrate as the nitrogen source whereas it was highest in the cytoplasmic fraction of cells harvested from media with glycine as the nitrogen

source. Gelatinase zymography showed that the cytoplasmic fraction of cells grown in Sauton's media with ammonium chloride and nitrate as nitrogen sources contained the highest activities. The zymograms showed two distinct bands corresponding to approximately 120 kDa and 70 kDa and two minor bands at 48 kDa and 20 kDa. In the CFP fraction, one minor band was visible only in the medium with nitrate as a nitrogen source, corresponding to approximately 50 kDa in size. Additionally, seven monoclonal antibodies specific for seven distinct antigens of *M. tuberculosis* were used to screen for cross reactivity with the secretory fractions of *S. albus.* Of the seven antibodies, only one (F181-ID3-2) gave a positive reaction. This is a monoclonal antibody directed at a specific internal amino acid sequence in the secreted acid phosphatase of mycobacteria (SapM). This protein has a size of about 28kDa and is implicated in the pathogenesis of *M. tuberculosis.*

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List of Abbreviation

M	Molar
mmol	Millimoles
µL	Microliter
µg	Microgram
mM	Millomolar
Abs	Absorbance
cm	Centimeters
EDTA	Ethylenediaminetetracetic acid
DNA	Deoxyribonucleic acid
h	Hours
kDa	Kilo Dalton
L	Liter
LB	Luria Bertani
mg	Milligram
min	Minutes
ml	Milliliters
MW	Molecular weight
nmol	Nanomole
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
rRNA	Ribosomal ribonucleic acid

RT	Room temperature
sec	Second
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
BCG	The live bacille Calmette-Guérin
TB	Tuberculosis
MTB	*Mycobacterium Tuberculosis*
S. albus	*Streptomyces albus*
Streptomyces SP.	Streptomyces species
HIV	Human immunodeficiency virus
PPD	Purified protein derivative
IFNγ	Interferon gamma
TNFα	Tumor necrosis factor alpha
NO	Nitric oxide
YM	Yeast Malt
TSB	Tryptic Soy Broth
TCA	Trichloroactetic acid
Tris	Tris(hydroxymethyl) aminomethane
TEMED	Tetramethylethylenediamine
bp	Base pairs
U	Unit
V	Volume
pNPP	p-Nitrophenyl Phosphate
NAD	Nicotinamide adenine dinucleotide

TBST	Tris buffered saline contains Tween
rpm	Revolutions per minute
AmCl	Ammonium chloride
Asp	L-Asparagine
Nit	Nitrate
Gly	Glycine

1. Introduction

1.1 Tuberculosis: The Disease

Infectious diseases remain one of the leading causes of death worldwide (Morens, eat al., 2004). Infections can be caused by parasitic, fungal, viral and bacterial pathogens. Each year, approximately 17 million deaths are caused by bacterial infections alone (Khaliq et al., 2012). It has been estimated that up to one third of the global population are infected with *Mycobacterium tuberculosis*, the causative agent of Tuberculosis (TB) (Schluger and Rom, 1989).

Tuberculosis is a fatal disease and considered as the seventh leading cause of death in the world comparable to AIDS and malaria (WHO, 2004, Causes of death). It is highly associated with human immunodeficiency (HIV), which is determined as the global co-infection of *M. tuberculosis* (Toossi et al., 2001). HIV works to reactivate the infection of latent *M. tuberculosis* (MTB), rapidly raises the danger of the infection, and re-activates MTB (Corbett et al., 2003). Therefore, the spreading of HIV epidemic and the development of multi-drug resistant strains of *M. tuberculosis* (MDRTB) is increasing which makes the improvement of TB control greatly needed (McShane et al., 2005). In 2012, the incidence of TB was 58 % in Asia and 27% in African regions, while the lowest incidence were recorded in Eastern Mediterranean regions (8%), in Europe (4%), and in North American (3%) (WHO, 2013). TB is primarily a respiratory disease affecting the lung but can spread from this initial site to other parts of the body producing a disseminated infection. During active disease, the pathogen spreads to others through droplets released during exhalation and/or coughing (WHO, 2013). The most striking

symptoms of the disease include coughing, weight loss, and fever (Moreno et al., 1989). In late stage acute disease, blood is observed in mucous released through severe coughing (Moreno et al., 1989).

Early diagnosis of infection and direct application of suitable chemotherapy provide the best TB prognoses. The standard battery of diagnostic tests includes detection of the acid-fast bacilli in sputum, clinical indicators, and X- ray radiographic imaging of the lungs (Brust et al., 2011). Due to costs and other factors, such as the availability of proper clinical equipment, these tests are often not prescribed initially. Instead, the standard is to use the tuberculin skin test with purified protein derivative (PPD) antigens. This test involves simply injecting the PPD, which is prepared from sterilized spent culture media of the pathogen, subcutaneously in the forearm. Following 24 hours after injection, the site is monitored for a delayed-type hypersensitivity reaction and the diameter of the reaction is used to determine the result (Figure 1) (Chapman et al., 2002) (Brust et al., 2011). A diameter of less than 10 mm is considered negative, between 10-14 is considered uncertain, and larger than 14 mm is considered positive (Farhat et al., 2006). This test remains the most widely accepted initial diagnostic for the detection of active disease or for previous exposure to the pathogen. This test however has several draw backs such as the positive reactions often seen with health care workers, the fact that previously vaccinated individuals will also test positive and finally the length of time it

Figure 1. The Tuberculin or PPD test for the initial diagnosis of TB. An injection of the PPD preparation is injected under the skin in the forearm. The diameter of the hypersensitivity reaction at the site of the injection is used to determine the test result. Reproduced with permission from: Giant Mantoux reaction, by Avasthi, R., Chaudhary, S. C., and Mohanty, D. (2009) Indian journal of medical microbiology, 27(1), 78-79.

takes to arrive at a conclusion. There are presently a number of alternative tests in use particularly in Europe and North America. A test that appears to be gaining popularity is the ELISA-based interferon test (The enzyme-linked immunosorbent assay). It is a blood test that detects latent TB by measuring the release of interferon (IFN)-γ by lymphocytes when exposed to antigens of *Mycobacterium tuberculosis* (Lalvani and Pareek, 2010).

1.2 *Mycobacterium tuberculosis*

1.2.1 The Pathogen

In 1882 Robert Koch published the first of two reports on tuberculosis and he was the first one to implicate the pathogen, *M. tuberculosis*, as the causative agent for the disease (Daniel, 2006). Mycobacteria is a genus of the actinobacteria, the largest phylum amongst the bacteria, containing Gram-positive bacteria with genomes rich in Guanine and Cytosine (GC rich) (Ventura et al., 2007). Physiologically, members of this genus are considered aerobes and thus require high level of oxygen to multiply, making the lung a good target for infection. *M. tuberculosis* is an acid-fast bacillus (Flynn, 2004) requiring specialized staining methods for microscopic examination and is resistant to many antibiotics (Johnson et al., 2006). This resistance is not acquired but is due to the nature of the cell wall of the pathogen. To illustrate, the outer surface of the pathogen contains mycolic acids with hydrocarbon chains as long as 70 carbons (Liu et al,. 1995). Mycolic acids are lipid-rich that make the cell wall thick and complex providing an extremely efficient permeability block (Hoffmann et al., 2008). This protects the bacillus against the body's immune response, so staining this bacterium is difficult and protects the pathogen from drying outside the host for long period of time

(Rao and Meena, 2010). It is this feature that makes the pathogen resistant to many antibiotics as this waxy surface layer limits diffusion of the antibiotics towards their targets inside the cell. There are currently four antibiotics that are used in combination therapy for TB including: isoniazid, rifampin, pyrazinamide, ethambutol (WHO, 2005, *New Tuberculosis Therapy Offers Potential Shorter Treatment*) (Ginsberg and Spigelman 2007). Mycobacteria includes other important human and animal pathogens; *M. bovis* (cattle TB), *M. leprae* (leprosy), *M. marinum* (fish pathogen), *M. avium* (bird pathogen), and *M. africanum* (TB-like signs but less virulent). Some non-pathogenic mycobacteria include the vaccine strain *M. bovis* BCG and the commensal *M. smegmatis*, both of which are common surrogates in research for *M. tuberculosis* (Gengenbacher and Kaufmann, 2012).

1.2.2 Pathology and Immunology

The morphological characterization of Mycobacteria can be divided in to two different metabolic/morphological phases according to environmental conditions (immune response in this case). When the host immune response becomes weak, vegetative growth is initiated by the pathogen, which causes active Tuberculosis (active state). At late stage of infection, when the immune response is robust, host immune responses suppress the vegetative growth. This causes latent TB (dormant state) by inducing the persistence formation of bacillus, which can survive in this stage for years (Scherr and Nguyen, 2009). The majority of TB infected people have latent TB characterized by dormant bacilli and less than 1% develops the infection and get active TB (Hatzios and Bertozzi, 2011).

The infection with *M. tuberculosis* in humans starts by inhaling droplets having a small numbers of bacteria (Kaufmann, 2001). Once in the lung, alveolar macrophages engulf the bacilli by the process of phagocytosis. This is an important component of the innate immune response and is meant to neutralize and disintegrate the pathogen. This neutralization process unfortunately is terminated prematurely within the macrophage and the pathogen survives the phagocytic process. Subsequent movement of the macrophage to other locations within the body while harboring the bacilli leads to release of live bacilli to lymph nodes and other parts of the body. Those that remain in the lungs and kill host macrophages and other cells trigger an immune response that precipitates the formation of a clot to surround and contain the foci of the infection. With time, these transform into granulomatous lesions containing live but dormant bacilli. The disease may not develop directly because the bacterium is able to persist dormant for many years depending on the health status of the individual and other factors. To illustrate, the disease is developed directly after initial infection when the immune response is compromised such as by the co-infection with HIV, poor nutrition, or any other risk factors. The dormant infection can become activated at some point at a later time and cause a disease referred to as reactivation Tuberculosis (Gengenbacher and Kaufmann, 2012).

The mechanisms that play roles in the formation and deformation (fragmentation) of the granulomas are complex and involve several T cell populations. T cells play an important protective role in TB; these include $CD4^+$ and $CD8^+$ T cell with the profile of T helper type 1 cytokine (Hingley- Wilson et al., 2003). Additionally, two main mechanisms are associated with the acquired resistance of the immune system: macrophage activation

by cytokines and cytolytic activity. Secretion of interferon γ (IFNγ) by T cell populations is the significant mediator of macrophage activation and acts with the tumor necrosis factor α (TNFα) to stimulate the functions of anti-mycobacterial activity (Kaufmann, 2002). CD4+ T cells participate in protection against TB by producing lymphotoxin α (LTα) and CD8+ T cells kill the bacteria within macrophages by producing granulysin and perforin (Kaufmann, 2002). As the cells in the immediate area of these immune system reactions synthesize fibrous extracellular material to contain the insult granulomatous lesions are formed (Kaufmann, 2002).

1. 2. 3 Phylogenetic Classification of Mycobacteria

A phylogenetic tree is a genetic tool that constructs an evolutionary relationship between organisms (Lodders and Kämpfer, 2007). The most widely used method to identify and classify organisms is through the 16S ribosomal RNA (rRNA) gene sequence (Datta et al., 2012) because it is conserved in all organisms, it has an optimum size (1,500 bp), and invariable function (Janda and Abbott, 2007). Based on this type of analysis, a phylogenetic relationship has been described within mycobacterial species (Kim et al., 1999), a high relative similarity have been detected from 94.3% – 100% among the genus (Blackwood et al., 2000). To illustrate, some mycobacterial species match the same sequence, for example MTB complex members, *M. gastri* and *M. kansasii, M. farcinogenes* and *M. senegalense*, *M. ulcerans* and *M. marinum, M. szulgai* and *M. malmoense* (Devulder et al., 2005). Mycobacterium is also related to other bacteria such as the streptomyces (McGuire et al., 2012) (Figure 2).

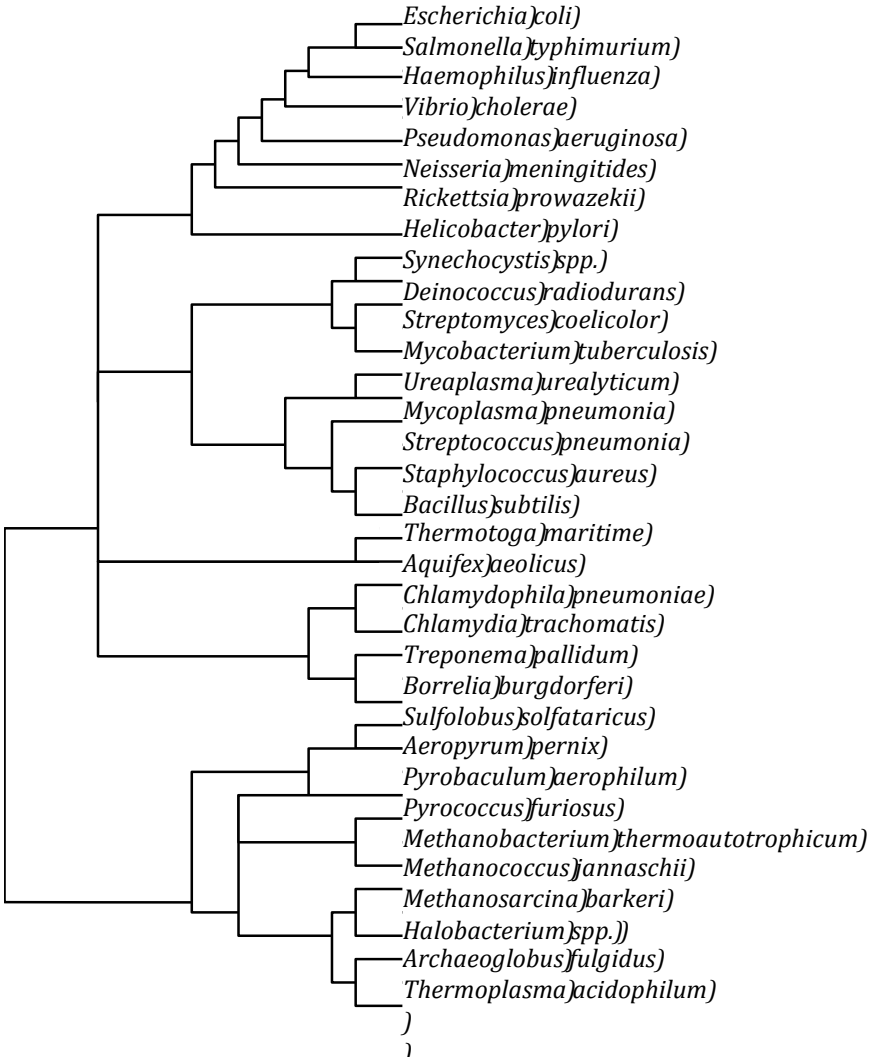


Figure 2. Phylogenetic classification of *Mycobacterium tuberculosis* based on 16*S* rRNA gene sequences. Adapted from "The origin and evolution of model organisms" by Hedges, S. B. (2002).

1.3 *Mycobacterium bovis* Bacille Calmette-Guérin (BCG)

In 1921, Bacille Calmette Guerin (BCG), derived from *M. bovis* because its similarity with *M. tuberculosis* in more than 90% of DNA homology (Andersen, 2001), it was used for protection against tuberculosis (Pym et al., 2003). BCG was derived from a cow infected by bovine tuberculosis (Orme, 2001), and developed by culturing the original isolation in different passaging and laboratories production condition of 230 successive passages (Mahairas et al., 1996). The Pasteur Institute of Paris distributed BCG vaccine in several countries using unstandardized culture conditions, so more than 14 BCG sub strains used as a BCG vaccine around the world (WHO, 2011, *Recommendations to Assure the Quality, Safety and Efficacy of BCG Vaccines*), are genetically, phenotypically, and in their vaccine properties different (Liu et al., 2009). BCG strains have been divided regarding to the mutations in the bacilli genome into early and late strains. In addition, the early strains, including BCG Moreau-RJ, BCG Russia, BCG Tokyo 172-1, BCG Birkhaug and BCG Sweden, have the same characteristics of the BCG Pasteur (the original strain) with less deletions and mutations in their genomes than the late ones. The late strains include BCG Glaxo (Copenhagen 1077), BCG Pasteur 1173P2, BCG Prague, and BCG Danish 1331 (WHO, 2011, *Recommendations to Assure the Quality, Safety and Efficacy of BCG Vaccines)*. These differences between the BCG strains were explained as being due to mutations caused by continued laboratory passaging of the parent *M. bovis* pathogen (Osborn, 1983).

A number of BCG vaccines are currently in use in various countries around the world and with various degrees of efficacies. The BCG vaccine may provide a certain level of protection when used at an early age, but not in all cases as pointed out earlier.

This protection decreases with age and typically disappears in adulthood (Reece and Kaufmann, 2008). Complicating this risk is the fact that individuals successfully treated for TB often develop re-activation TB, whereby un-killed bacilli that remained dormant during the treatment become active and cause disease at some point in the future (Verver et al., 2005, 2001). Researchers have investigated several approaches to create different and more efficient vaccines. Some of these approaches include the use of heterologous systems (transfer of proteins from one species to another). For example, the modified vaccinia ankara virus expressing antigen 85A (MVA85A) vaccine was used as a first heterologous enhancement for BCG protection in children who received BCG at birth (Scriba et al., 2010). It was shown that this vaccine was more efficacious, safer, and has great immunogenicity compared to BCG alone (Tameris et al., 2013). Different live microorganism (*M. microti, M. vaccae, and M. habana*) induces immunological memory and are used as mycobacterial antigens expressing vectors. Not one of these non-pathogenic mycobacterial vectors shows better protection than BCG. Non-mycobacterial organisms also used to give high rate of protection such as Salmonella, but they had many problems (Arzuaga et al., 2011). Streptomyces has been recommended as a candidate for vaccine development against TB because it is phylogenetically related to mycobacteria and is well established for heterologous production of proteins (*S. lividans*) for several years (Vallin et al., 2009)

1.4 Streptomyces

Streptomyces was discovered by Waksman and Henrici in 1943 (Anderson and Wellington, 2001), and it is one of the actinomycetes that are responsible for producing around 70% of the 22,000 recognized bacteriological secondary metabolites (Subramani and Aalbersberg, 2012). This soil bacterium is industrially used for heterologous protein production (Schaerlaekens et al., 2004), and has the greatest number of species when compared to other bacteria and archaea. Moreover, it is a Gram-positive bacterium with high guanine and cytosine content in its genome and possesses cell wall composed of cytoplasmic membrane surrounded by a mesh of peptidoglycan (Scherr and Nguyen, 2009). Phylogenetically, it is related to the mycobacteriaceae family (Vallin et al., 2009).

The morphological phase of streptomyces life cycle is distinct and complex. It is similar to that of filamentous fungi, both organisms form vegetative mycelium. In addition, the developmental stage of the filamentous streptomyces depends on the environmental conditions (antibiotic biosynthesis). If it is favorable, multi-genomic hyphae will be produced by substrate mycelia. At late stages when conditions are unfavorable these hyphae septate into spores (Flärdh and Buttner, 2009).

Streptomyces are amongst the highest producers of bioactive secondary metabolites such as various antivirals, antifungals, anti-hypertensive, immunosuppressant, and antibacterials (Jensen et al., 2007). Indeed, 75% of medical and commercial antibiotics are produced by different species of streptomyces (Ceylan et al., 2008). Production of a number of secondary metabolites coincides with the morphological

differentiation of these bacteria; the production of antibiotics in liquid cultures is normally restricted to the stationary stage (Tieleman et al., 1997). Although streptomyces are well known for their antibiotics production, few of these filamentous species have pathogenic strains to plants and animals (Lodders and Kämpfer, 2007). *Streptomyces sp*. causes mycetoma: skin and tissue infections and other infections that are described as invasive infection (Kapadia et al., 2007). These infections involves disseminated and bacteremia diseases, infections of respiratory tract and the central nervous system (Datta and Arora, 2012). Immunocompromised patients are the most vulnerable to these infections for example whether with HIV infection or on immunosuppressives (Datta and Arora, 2012).

Spores of molds found in damp structures have been shown to induce the production of cytokines and nitric oxide (NO); both recognized as inflammatory mediators causing respiratory tract syndromes (Hirvonen et al., 1997). However, spores of streptomyces that are typically found in moldy houses shows highly increased production of these mediators comparing to other microorganisms spores found in the moldy buildings (Hirvonen et al., 1997) and the effective mechanisms are poorly understood (Jussila et al., 1999). In addition, the production of NO in the airways plays an important role to promote inflammation, tissue damage, edema, vasodilatation, and cytotoxicity (Hirvonen et al., 2005). Nitric oxide NO is also responsible for macrophage activation, which can kill the bacteria and cytokines production such as TNFα and IL-6, which cause allergic inflammation (Hirvonen et al., 1997). Moreover, streptomyces create valinomycin in the indoor dust. Valinomycin is a toxin that inhibits the Function of human NK cell, which is regulated by

Interleukin-18 (IL-18) and Interleukin-15 (IL-15) cytokines. NK cells are response for cytokines secretion such as IFNγ and TNFα to active the immune response (Paananen et al., 2000). For instant, *S. somaliensis* is a pathogenic strain and the major cause of actinomycotic mycetoma (Kirby et al., 2012). Mycetoma infects tissue and bone deeply infection (Kirby and Sangal 2012). It infects men more than women particularly those 20 to 40 years old (Fahal 2004). It is considered as a fatal disease because it destroys and deforms the tissue by forming tissue masses (Kirby and Sangal 2012). The most affected parts of the body are feet (Fahal 2004) and it characterized by swelling, drainage and nodule development (Lichon and Khachemoune, 2006). *Streptomyces albus* is another species that is widely recognized as an appropriate host for the heterologous production of bioactive secondary metabolites (Zaburannyi et al., 2014). It causes a human disease called hypersensitivity pneumonitis or HP (Kagen et al., 1981). HP happens because of sensitization to antigens that are derived from organic materials such as bacteria (Kupeli et al., 2010). Peritonitis is a non- mycetomic (invasive) infection caused by streptomyces species such as *S. viridis* and it demonstrated by fever, abdominal swelling, and discomfort in the abdomen (Datta et al., 2012). Potato scab is an important disease that caused by plant pathogenic species of streptomyces (Loria et al., 2006): *S. acidiscabies, S. ipomoeae, S. scabiei,* and *S. turgidiscabies* (Han et al., 2005). Superficial and raised lesions on tuber surface characteristic symptoms of common scab of potato (Han et al., 2005). In summary, the infections caused by *Streptomyces sp.* are uncommon and there are no breakpoint antibiotics for this pathogen. The common drugs used include TS, amikacin, dapsone alone or combined with TS, and amoxicillin-clavulanic acid (Martín et al., 2004).

1.5 Research Rationale

The current vaccine against TB is the live Bacille Calmette-Guérin (BCG) vaccine. It is not effective, particularly in adult populations, and current efforts are directed at investigating new approaches for a better vaccine. Several different approaches to create such a vaccine were investigated at various laboratories and included DNA vaccines, subunits vaccines, and heterologous systems (salmonella) but with little success. Considering the phylogenetic relatedness of mycobacteria and streptomyces, the latter may provide an alternate strategy to develop a heterologous carrier of *M. tuberculosis* antigens as a new vaccine. As an initial step towards this goal, an enzymatic and immunological profiling of streptomyces will be valuable. Since the interaction of a pathogen with its host determines the outcome of an immune response it will be useful to compare these physiological and immunological interactions between both microbes and the host. The more similar their interaction with the host the more similar the immunological response will be. For this to be likely however, both microbes must be capable of producing similar types of enzymes that would interact with the host in similar ways. Furthermore, immunological profiling of proteins secreted by both microbes would uncover any similarities in terms of antigen presentation during the interaction with the host. In support of this notion, the genome of *S. coelicolor* was compared recently with those of two pathogenic mycobacterial species: *M. tuberculosis* and *M. bovis*. It showed a high similarity at the individual gene sequences level: 740 (18.56%) in *M. tuberculosis* and 761 (19.42%) in *M. bovis* genes have over 50% homology with genes from *S. coelicolor* (Arzuaga et al., 2011). In support of these reports, a BLAST search using SapM (secreted acid phosphatase of mycobacteria) amino acid sequence against predicted

proteins of streptomyces results in the detection of several homologs with over 50% sequence homology. One such homolog is presented in Figure 3. These are bioinformatic studies and one must show that these similarities exist at a functional level. This study was conducted to see if a pathogenic streptomyces (a clinical isolate related to *S. albus*) indeed synthesizes these gene products by testing for the activities of several enzymes secreted to the culture media and by testing for cross reactivity of antibodies directed at *M. tuberculosis* antigens with these gene products.

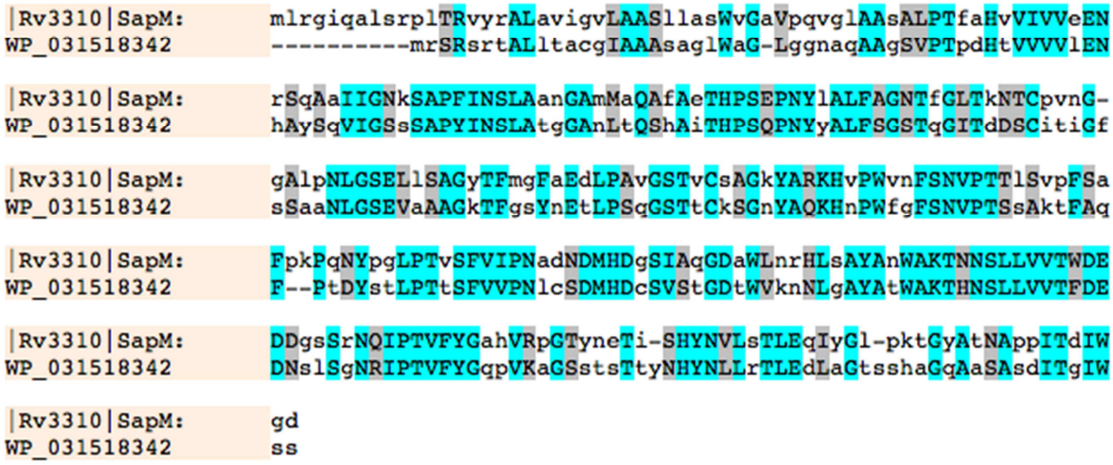


Figure 3. Sequence alignment of Rv3310 (SapM) and a streptomyces homolog.

1.6 Research objectives:

The main research objective of this study is to establish a functional relationship between *M. tuberculosis* and *S. albus* using enzymatic and immunological profiling. This will confirm that the relatedness of the two bacteria extends beyond phylogenetics and bioinformatic analysis and may include multiple antigens of value in vaccination. The specific objectives are:

(1) Evaluate the levels of 19 enzyme activities in cellular fractions of a clinical isolate (*S. albus*) of streptomyces. These will be compared with the enzymatic profile of *M. tuberculosis* established several years ago by another laboratory. This number of enzymes was selected because of the availability of a commercial product (*API ZYM*e, Biomerieaux, QC) that can provide qualitative analysis of 19 enzyme activities. It is a large enough number to draw conclusions on common activities found in *M. tuberculosis* and *S. albus.*

(2) Evaluate the cross reactivity of eight monoclonal antibodies specific for antigens of *M. tuberculosis.*

2. Materials and methods

2.1 Materials

The streptomyces clinical isolate was kindly provided by the Public Health Agency of Canada on September 2011 and was marked as *S. albus* strain NML no.05-0087. Monoclonal antibodies (NR-13607, NR-13804, NR-13605, NR-13817, and NR-13793) were provided by bei Resources (a program of the National Institute of Allergy and Infectious Diseases, VA, USA). The internal peptide (DNDMHDGSI) of SapM was synthesized by GenScript (Piscataway, NJ, USA) and was used to generate the monoclonal antibodies ANTI-4287-F181-ID3-2, ANTI-4287-F181-4C2-2, and ANTI-4287-F181-5F12-2. The antibodies were produced by the National Research Council of Canada (NRC, Ottawa, Canada). Yeast Malt (YM) extract, Peptone, tryptic soy broth, agarose, acrylamide, sodium dodecyl sulfate, trishydroyxmehtylaminoethane (TRIS), ethidium bromide, ethylenediaminetetraacetic acid (EDTA), tetramethylenediamine (TEMED), ammonium persulfate, bromophenol blue, Coomassie Brilliant Blue R, methanol, and acetic acid were purchased from Sigma-Aldrich CO. (St. Louis, MO, USA). Ferric Ammonium Citrate, Ammonium chloride, L-Asparagine, potassium phosphate, magnesium Sulfate, glycine, citric acid, and potassium nitrate were purchased from Thermo Fisher Scientific Co. (Waltham, MA, USA). All other supplies and reagents were purchased from Sigma-Aldrich CO. (St. Louis, MO, USA).

2.2 Methods

2.2.1 Culture Media

Solid media used for routine culturing of the clinical isolate was the Yeast Malt (YM) agar prepared according to the supplier's instructions. For culturing and passaging in liquid media, two types of media were used: tryptic soy broth (TSB) and Sauton's media (minimal media typically used to culture mycobacteria). Sauton's media was prepared as described by Chen et al. (2003).

2.2.2 Culture Technique

Sauton's media was the choice of liquid media for this work because it is a defined medium with no protein sources, thus facilitating enzyme assays and establishing growth medium for future proteomic work. The clinical isolate was initially inoculated into TSB medium from the original plate and incubated for a week at RT. A loopful was then spread on YM agar plates having 100 μg /ml ampicillin and incubated at 3°C until colonies became visible (24 hours). A colony was selected as the stock pure culture and used for all subsequent growth experiments.

2.2.3 Strain Identification

2.2.3.1 Genomic DNA extraction

Cells cultured in 30 ml medium were pelleted for 5 min at 4000x*g* and washed twice with 10 ml 10% Sucrose. Then it was mixed with 10 ml lysis buffer. Ten milligram lysozyme also was added to the mixture and incubated at 37°C for 20 min. That was followed by another incubation in a water bath at 55 °C for 1.5 h with addition of 1.0 ml

10% SDS and 5 mg proteinase K. A volume of 3.6 ml of 5 M Sodium chloride and 15 ml of chloroform were added and the mixture rotated end over end for 20 min at 6 rpm. Once more, centrifugation was done at 5000x*g* for 2 minute, and the supernatant was divided into three separate phases, the top phase was the only phase used and it was transferred to a new 50 ml Falcon tube with 8 ml 100% isopropanol. DNA appeared after few minute and transferred to a new microfuge tube containing 1 ml 70% ethanol, centrifuged at 13,000 rpm for 10 min, ethanol removed and dried DNA completely. Depending on pellet sizes, pre-warmed buffer was added and the DNA resuspended using a pipette. DNA was quantified using a Shimadzu spectrophotometer with a built-in program for calculating the 260 nm /280 nm ratio.

2.2.3.2 PCR amplification

Two sets of PCR primers were designed to amplify fragment of the 16*S* ribosomal RNA gene. The forward primer sequence: GCAATGCTGGCGGCGTGCTTAACACATG and the reverse primer sequence TTGCCCAATCGCCAGTCCCACCTTCG (supplied through Invitrogen). PCR amplification was performed in a 50 μL reaction containing: 1 μg DNA template, 5 μL 10x Dream Tag^{TM} buffer, 5 μL of 0.2 μM dNTP mix, 1.25u Dream Tag^{TM} DNA Polymerase, 1.0 μM of both reverse and forward primers, and 30.25 μL nuclease free water. PCR was carried out with primary denaturation for 3 min at 94 °C and that followed by 31 cycles of: 30 sec at 94 °C, 1:30 min at 65 °C, 1 min at 72 °C, with a final incubation at 72 °C for 10 min. The PCR product was subsequently purified from agarose gels using QIAquick spin columns (QIAGEN GmbH, Germany).

2.2.4 Protein analysis

2.2.4.1 Protein extraction

The culture was divided between culture filtrate protein (CFP) and whole cell lysate (WCL) using 50 ml centrifuge tubes at 4500 rpm for 15 min in Beckman GS centrifuge. Centrifugal filter devices with UItracel YM -10 membranes were then used to concentrate the culture supernatant. The concentrate (3-5 ml) was dialyzed in dialysis tubing (Spectrum) against 1.0 L of 2 mM ammonium Bicarbonate at 4°C overnight. Whole cell lysate was prepared by resuspending the pellet in 5 ml distilled water and subjected to 5 cycles of 10 sec ON/10 sec OFF of sonication using a Heat Systems Ultrasonics (W-220F) cell disruptor while on ice. Protein in the extracts was quantitated using the Pierce BCA protein assay kit (Thermo Fisher Scientific CO. Waltham, MA, USA).

2.2.4.2 Protein Separation by Electrophoresis

Proteins of the extracts were concentrated by acid precipitation and were subsequently separated into individual components using standard SDS-PAGE. Briefly, 1 volume of 100% (w/v) Trichloroacetic acid (TCA) was add to 4 volumes of protein sample followed by 4°C incubation for 10 min. Then the mixture was centrifuged for 10 min at 14,000 rpm to pellet the proteins. The pellet was washed three times with 200 µL cold acetone and the residual acetone removed by placing the tube in 95°C heat block for 5-10 minutes. The protein samples were finally separated by SDS-PAGE using a vertical mini-PAGE electrophoresis system for 90 min at 200 V and 20 mAmp.

2.2.5 Enzyme Activity Assays

2.2.5.1 *API ZYM* activity assay

The *API ZYM* system (Biomerieux. QC, Canada) is a ready to use multi enzyme assay system which requires only the addition of a small volume of the test media into micro wells (cupules) containing the appropriate chromogenic substrate (Table 1). It is a rapid and semi-quantitative test of 19 enzyme activities. The samples were added to each cupule of the strep by different volume according to the protein concentration (500 µL of dissolved protein) sample, placed in the dark, and incubated for 45 min at room temperature. Then, 20 µL of Fast Garnet GBC buffer was added as a developer, incubated for four hours. The outcome reactions were read according to the reading table (*API ZYM* – REF 25 200).

2.2.5.2 Phosphatase Assay

For quantitative analysis, alkaline and acid phosphatase activities were assessed by the release of the yellow colored *p*-nitrophenol (*p*NP) from *p*-nitrophenyl phosphate (*p*NPP) through its hydrolysis. The assay was carried out in a total of 200 µL reaction volume: 20 µL protein sample, 20 µL 20 mM *p*NPP (Fermentas. Ottawa, Canada), and 160 µL 0.1 M Tris-HCl , buffer pH 6.5 (acid phosphatases), pH 9.6 (alkaline phosphatases), incubated at 37 °C for 60 minutes followed with the absorption measurement of the reactions at 405 nm using a FLUOstar OPTIMA version 1.30.0 (BMGLABTECH). For calculation of specific activities a molar absorption coefficient of 13,800 M^{-1} cm^{-1} for *p*-nitrophenol was used (Rashamuse, et al., 2009).

Table 1. A list of substrates utilized in the *API ZYM* test to detected enzymes activities. Adapted from Adelantado et al. (2007).

Enzyme assayed	substrate
Control	-
Alkaline phosphatase	2-naphthyl phosphate
Esterase (C 4)	2-naphthyl butyrate
Esterase Lipases (C 8)	2-naphthyl caprylate
Lipase (C 14)	2-naphthyl myristate
Leucine arylamidase	L-leucyl-2-naphthylamide
Valine arylamidase	L-valyl-2-naphthylamide
Cystine arylamidase	L-lcystyl-2-naphthylamide
Trypsin	N-benzoyl-DL-arginine -2-naphthylamide
α-chymotrypsin	N-glutaryl-phenylalanine-2-napthylamide
Acid phosphatase	2-naphthyle phosphate
Naphthol-AS-BI-phosphohydrolase	Naphthol-AS-BI-phosphate
α-galactosidase	6-Br-2-naphthyl-αD-galactopyranoside
β-galactosidase	2-naphthyl-βD-galactopyranoside
β-glucuronidase	Naphthol-AS-BI-βD-glucuronide
α-glucosidase	2-naphthyl-αD-glucopyranoside
β-glucosidase	6-Br-2-naphthyl-βD-glucopyranoside
N-acetyl-β-glucosaminidase	1-naphthyl-N-acetyl-βD-glucosaminide
α-mannosidase	6-Br-2-naphthyl-αD-mannopyranoside
α-fucosidase	2-naphthyle-αL-fucopyranoside

2.2.5.3 Alanine Dehydrogenase Assay

The activity of this enzyme was assayed by measuring the production of NADH from NAD^+ according to the method described by Ohashima and Soda (1979). The reaction mixture contained 100 μL protein sample, 200 μL H_2O, 100 μL 1.0 M L-alanine, 100 μL 500 mM glycine/KCl buffer (pH 10.5), and 500 μL 4.0 mM NAD^+. The absorbance was followed for 60 sec, at 30 °C and 340 nm using a Shimadzu UV spectrophotometer (Hitachi, Tokyo, Japan).

2.2.5.4 Alcohol Dehydrogenase Assay

For quantitative analysis, alcohol dehydrogenase activities determined by measuring the reduction of NAD^+ to NADH according to the protocol of Blandino et al. (1997). This assay is performed by assembling a reaction of 100 μL protein sample, 200 μL H_2O, 100 μL 1.0 M Tris- HCl buffer pH 8.8, 100 μL 100% ethanol, and 500 μL prepared fresh 4.0 mM NAD^+. The reaction mixture was incubated for 60 sec at 30 °C and the absorption change was read at 340 nm using a spectrophotometer. The specific activity was collected using the molar extinction coefficient of 6.22 L/mmol-1.0 cm for NADH.

2.2.5.5 Peroxidase Assay

For the activity of this enzyme, a fast and a qualitative test comprises mixing the protein sample with a solution of 6% hydrogen peroxide. Liberation of air bubbles, indicative of degradation of hydrogen peroxide and generation of oxygen gas, constitutes

a positive test.

2.2.5.6 Catalase Assay

The conversion of hydrogen peroxide to oxygen and water was done according to the method explained by Beers and Sizer (1952) by mixing 1.0 ml 0.059 M hydrogen peroxide, 1.9 ml H2O and 0.1 ml protein sample. At 240 nm and 30 °C, the absorbance was read for 60 sec by a Shimadzu UV spectrophotometer (Japan). For calculation of specific activities a molar absorption coefficient of 43.6 M-1 cm-1 for peroxidase was used (Saczko et al., 2002).

2.2.5.7 Gelatin Zymography

A qualitative assay of gelatinase activity employed gelatin zymography as described by Kleiner and Stetlerstevenson (1994). Protein samples (concentrated without acid precipitation and without heating or including reducing agents) were separated using standard SDS-PAGE except that gelatin at 1% final concentration was included in the acrylamide gel. Following electrophoresis, the proteins was soaked in 100 ml of 2.5% Triton X-100 in distilled water. In order to refold the separated protein, the solution was replaced with enzyme buffer and incubated overnight at 37 °C. The following day, the gel was stained in Coomassie Brilliant Blue R-250 and destined in water: methanol: acetic acid (50:40:10) solution to visualize the active bands in the gels.

2.2.6 Western Blots

Following electrophoresis, proteins were transferred onto nitrocellulose

membranes using transfer buffer on a BioRad Trans- Blot cell for an hour at 25 V and 200 mA. When the transfer is completed, the membrane was incubated in a blocking solution of 5% skim milk in TBST. The monoclonal anti-*M. tuberculosis* antibodies (Table 2) were used as primary treatment in appropriate dilutions in TBST buffer, and incubated for approximately an hour at room temperature. The membrane was then rinsed in TBST three times (10 minutes each at room temperature) and incubated with the secondary antibody[Anti-Mouse IgG-alkaline phosphatase] (Sigma). Once more, the membrane rinsed three times with TBST for 10 min each. Finally the membrane was kept in a premixed BCIP/NBT solution (Sigma. Oakville, Canada) for 10- 15 min to develop colour intensity and distilled water was used to stop the reaction.

2.2.8 Statistical analysis

Significance of variance was determined for triplicate measurements using one-way ANOVA.

Table 2. A list of the primary antibodies used in the western blots.

Antibody	Antigen
ANTI-4287, F181-ID3-2	Rv3310 SapM
ANTI-4287, F181-4C2-2	Rv3310 SapM
ANTI-4287, F181-5F12-2	Rv3310 (SapM)
NR-13607	Rv2031c HspX (α-crystallin)
NR-13804	Rv0475 (iron-regulated heparin-binding hemagglutinin (HBHA))
NR-13605	Rv0934 (periplasmic phosphate-binding lipoprotein)
NR-13817	Rv1860 (MPT32))
NR-13793	Rv1908c (catalase-peroxidase)
ANTI-ALADH, F181-5D6-2	ALAD

3. Results

3.1 Confirming the identity of the streptomyces clinical isolate being used in the experiments

Optimization of the PCR amplification reaction produced a unique band equivalent to about 1500 bp in size (Figure 4). The primers selected for this experiment were based on a report in the GenBank that described the production of a 1444 bp rRNA fragment from the genomic DNA of streptomyces as a template. The PCR fragment obtained in this study is the appropriate size of approximately 1500 bp. Following purification and sequencing of this PCR fragment, a sequence belonging to *S. albus* was obtained. This confirmed the identity and purity of the isolate.

3.2 Optimization of growth media and morphological characterization of *S. albus*

The composition and pH of growth media can affect the physiology and morphology of microorganisms. Literature reports on growth conditions for streptomyces are scarce and no reports exist on how growth media and pH affect enzyme production in these microorganisms. To ensure that the most diverse of enzyme production conditions are covered in this study, a number of different media were investigated. *S. albus* colonies in the presence of 100 µg/ml ampicillin grew best on YM agar plates during an overnight incubation at 35 °C (Figures 5 and 6). For the TSB liquid medium, growth became visible after a week at 35 °C in a shaker (Figures 7 and 8).

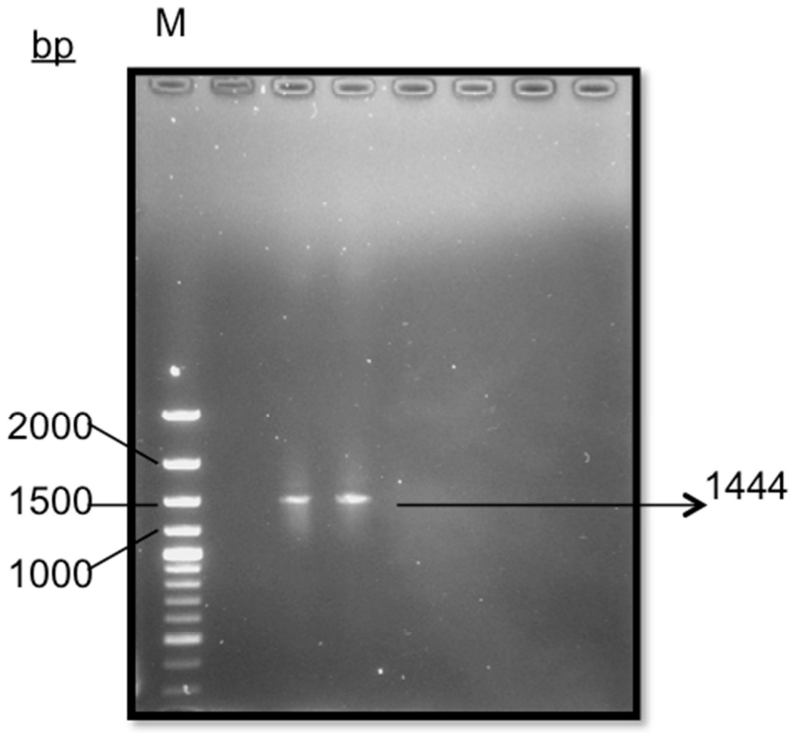


Figure 4. PCR amplification of the 16S rRNA gene fragment using purified genomic DNA of the streptomyces clinical isolates. A unique band close to 1500 bp was obtained and matches the expected size of the 1444 bp fragment expected using the primers designed in this experiment.

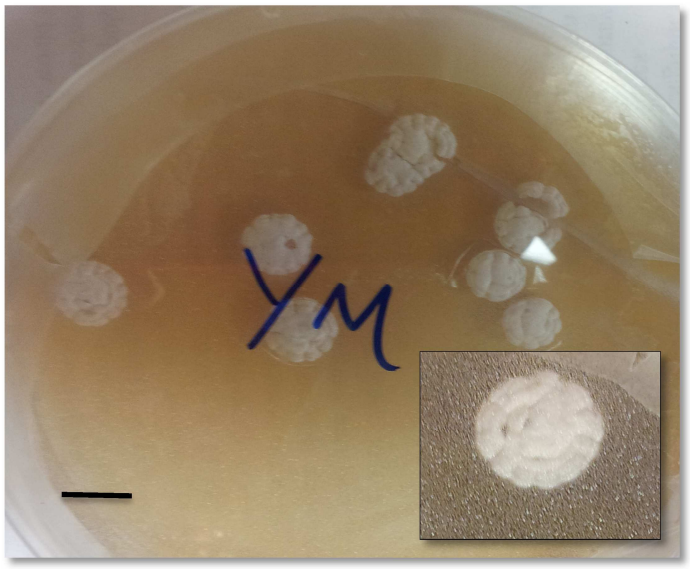


Figure 5. Colony morphology of *S. albus* on YM agar. The clinical isolate was initially inoculated into TSB medium from the original plate and incubated for a weak at RT. A loopful was then spread on YM agar plates having 100 μg /ml ampicillin and incubated at 30 °C (bar: 10 mm).

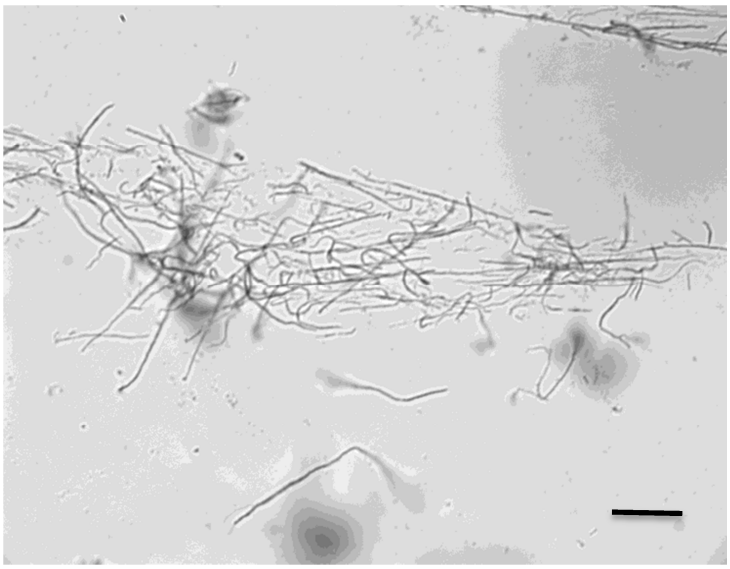
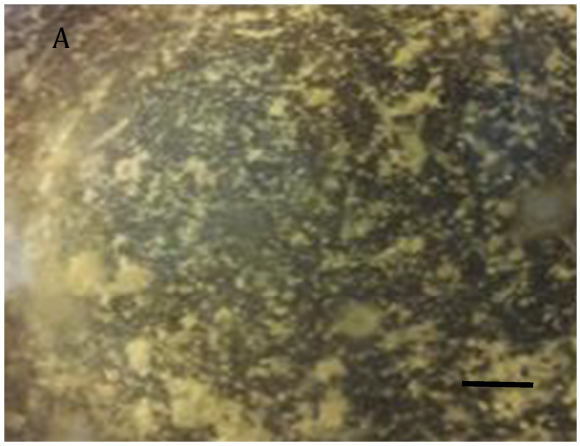


Figure 6. Air mycelia of *S. albus* as observed at 400x magnification (bar: 25 μm).



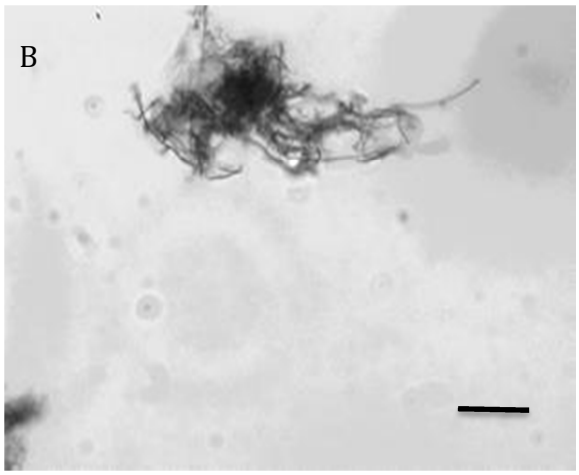


Figure 7. *Streptomyces albus* grown in Tryptic Soy Broth medium. The growth was under conditions of non-shaking and at room temperature. (A) Macroscopic image of the culture (bar: 10 mm). (B) A microscopic image (200x) for the same culture stained with Toluidine Blue (bar: 50 μm).

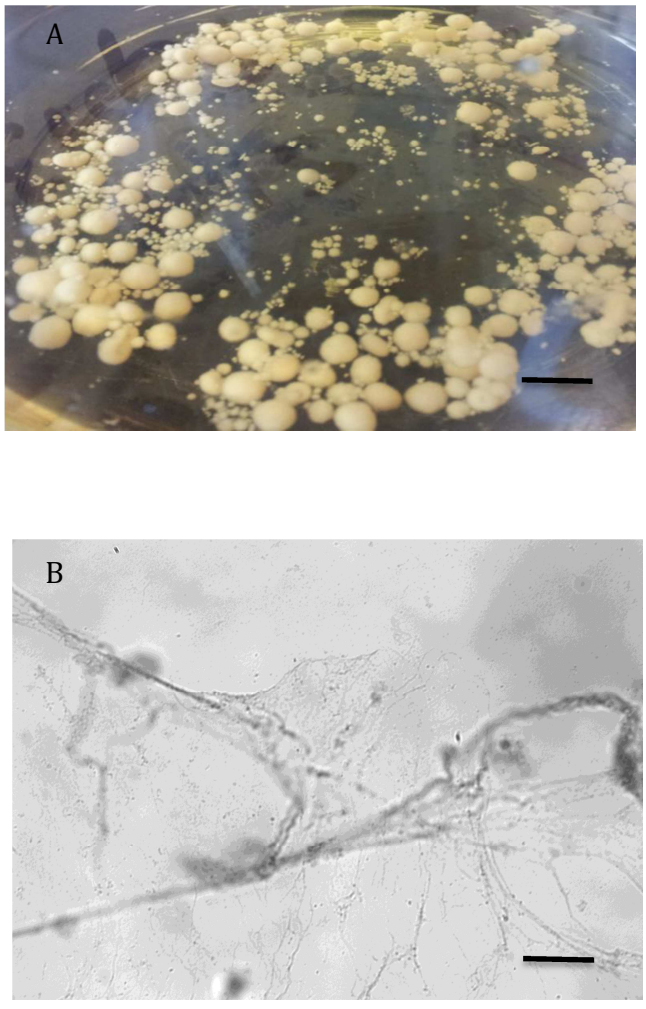


Figure 8. *Streptomyces albus* grown in Tryptic Soy Broth medium. The growth was under conditions of shaking and at 37°C. (A) A macroscopic image of the culture (bar: 10 mm). (B) A microscope image of the same culture stained with Toluidine Blue pigment; magnification 400x (bar: 25 μm).

As it is known that glucose is the best energy source for organism, rapid growth was notable in Sauton's medium containing glucose and ammonium chloride (as a nitrogen source). The growth was evaluated 2 days post inoculation and was compared with that from media in which ammonium chloride was replaced with glycine as a nitrogen source. It was notable that the growth in media containing the glycine was much slower (Table 3). However, no growth was detected of *S.albus* in Sauton's medium with glycerol and ammonium chloride while slow growth was notable in the one with L-Asparagine through two months from time of inoculation with shaking at 37 °C. To facilitate analysis of enzyme activities in the culture supernatant of this bacterium and to prepare conditions for future proteomic analysis of secreted proteins a defined simple medium that is protein-free is needed. Since streptomyces is a phylogenetic close relative of mycobacteria, a protein-free simple medium used in mycobacterial research is a logical first choice. Sauton's medium is considered a minimal medium with a defined carbon and nitrogen sources and a few other elements (see material and methods section 2.1 Culture Media). *S.albus* grew very well in a variety of Sauton's formulations (Table 3). Best growth was observed in Sauton's medium containing glucose as a carbon source and either ammonium chloride or sodium nitrate as nitrogen sources. This is in contrast to what is observed in mycobacteria however where the preferred carbon source is glycerol and the preferred nitrogen source is L-asparagine (Gouzy et al., 2014).

It has been reported that *Streptomyces sp*. prefer to grow in neutral to alkaline pH (Kontro et al., 2005). In Sauton's media, the initial pH normally adjusted to 7.4 before inoculation with the bacterium. Acidification of growth media is normal following

growth of bacteria as they produce a variety of organic acids as metabolic waste. This has been demonstrated in this study particularly in Sauton's media containing L-asparagine and ammonium chloride as nitrogen sources (Table 4). What is interesting however is that in *M. tuberculosis* and *M. bovis* BCG, acidification is seen only in Sauton's media containing ammonium chloride as a nitrogen source (M. Saleh, unpublished observations)? However, in Sauton's media containing L-asparagine, the pH of the spent culture medium actually becomes alkaline (M. Saleh, unpublished observations). Although this difference of pH may be explained by the different metabolic networks used in both groups of these bacteria, the significance is that acidification of the culture media implies that the bacteria will be experiencing a pH stress response and enzyme secretion may reflect that response. It is known for example that acid phosphatases are typically produced under acidic conditions (Pradel and Boquet, 1988).

Table 3. Optimization of growth conditions for *S. albus*. Different carbon sources (glycerol and glucose) and different nitrogen sources (L-asparagine, ammonium chloride, sodium nitrate, and glycine) were used to maximize growth of the pathogen. (+) Marked for how thick and fast was the growth while (-) marked for non-detectable result during that time.

	Medium	Growth
Glycerol - 2 months	L-Asparagine	Slow growth
	Ammonium chloride	-
Glucose – 2 days	L-Asparagine	+++
	Ammonium chloride	++++
	Sodium nitrate	++++
	Glycine	-

Table 4. Effect of growth of *S. albus* on media pH. The pH was measured for the four Sauton's media (with different nitrogen sources) following completion of the growth experiment. All cultures were in the stationary phase at the completion of the experiment.

Nitrogen Source	pH	
	Before	**After**
L-Asparagine	7.4	4.34
Ammonium chloride	7.4	5.84
Sodium nitrate	7.4	7.04
Glycine	7.4	7.02

3.3 Determination of streptomyces enzymes activities

3.3.1 Qualitative assays - *API ZYM* test

Activity of enzymes was quantified using *API ZYM*. During the incubation period, the products of the end metabolism produced and detected as color reactions. The outcome reactions were read according to the reading table (*API ZYM* – REF 25 200), the first cupule is the control, and always gives very pale yellow color, which is used as a measure of the cupules color results. An intense coloration of brown, red, and dark yellow or orange color was notable when positive reactions detected and the number of marks indicates increase of the coloration (Tables 5 and 6). With these different activity profiles of the spent culture media and cytoplasmic extracts, it was crucial to confirm the level of differences in the protein profiles amongst the different protein preparations. A simple SDS-PAGE profiling showed that indeed the compositions of these preparations were different (Figure 9, 10). For the Culture filtrate protein result, the medium with ammonium chloride has less bands comparing to others, but for the cytoplasmic protein, the less bands were as a result of medium containing L-Asparagine. All four spent culture media showed unique bands in terms of size as well as in terms of abundance.

Table 5. API-ZYM activity assay of *S.albus* culture filtrate protein. The samples each capsule of the strip by different volume according to the protein concentration, placed in dark, and incubated at 45°C, followed by another incubation with the developer (Fast Garnet GBC: [0.05 g fast Garnet GBC with 50 ml dH2O]) for four hours at 37°C. The number of marks indicates the intensity of coloration rate of enzyme reaction in the protein sample of the four Sauton's medium containing glucose and ammonium chloride (M), L-asparagine (S), sodium nitrate (N), or glycine (Y) as nitrogen sources. The number of marks (+) means increase of the coloration.

N	Enzyme	AmCl	Asp	Nit	Gly
1	Alkaline phosphatase	+++	+++	+++	+
2	Esterase (C 4)	-	+	+	-
3	Esterase Lipases (C 8)	+	+	-	+
4	Lipase (C 14)	+	-	-	+
5	Leucine arylamidase	++	++	+	++
6	Valine arylamidase	+	+	+	+
7	Cystine arylamidase	+	+	+	-
8	Trypsin	+	++	+	+
9	α-chymotrypsin	-	-	-	+
10	Acid phosphatase	+++	+++	+++	+++
11	Naphthol-AS-BI-phosphohydrolase	+++	+++	+++	+++
12	α-galactosidase	-	-	-	-
13	β-galactosidase	-	-	-	+++
14	β-glucuronidase	+	+	+	-
15	α-glucosidase	-	-	-	-
16	β-glucosidase	-	-	-	++
17	N-acetyl-β-glucosaminidase	+++	++	++	+++
18	α-mannosidase	-	-	-	-
19	α-fucosidase	-	-	-	-

Table 6. ZYM activity assay of *S.albus* cytoplasmic protein. The samples were added to each capsule of the strip by different volume according to the protein concentration, placed in dark, and incubated for 45 °C, followed by another incubation with the developer (Fast Garnet GBC: [0.05g fast Garnet GBC with 50 ml dH_2O]) for four hours at 37 °C. The number of marks indicates the intensity of coloration rate of enzyme reaction in the protein sample of the four Sauton's medium containing glucose and ammonium chloride (M), L-asparagine (S), sodium nitrate (N), or glycine (Y) as nitrogen sources. The number of marks (+) means increase of the coloration.

N	Enzyme	AmCl	Asp	Nit	Gly
1	Alkaline phosphatase	++	++	+++	++
2	Esterase (C 4)	+	+	-	-
3	Esterase Lipases (C 8)	+	+	-	-
4	Lipase (C 14)	+++	-	+	-
5	Leucine arylamidase	+	+++	++	+++
6	Valine arylamidase	+	+++	-	+++
7	Cystine arylamidase	+	+	+++	-
8	Trypsin	+	+++	-	-
9	α-chymotrypsin	+	+	+++	-
10	Acid phosphatase	+++	++	+++	+++
11	Naphthol-AS-BI-phosphohydrolase	+++	+++	-	+++
12	α-galactosidase	-	+	-	+
13	β-galactosidase	-	-	-	++
14	β-glucuronidase	-		-	-
15	α-glucosidase	-	-	-	+
16	β-glucosidase	-	-	-	+++
17	N-acetyl-β-glucosaminidase	-	+	-	+++
18	α-mannosidase	-	-	-	+
19	α-fucosidase	+	-	-	-

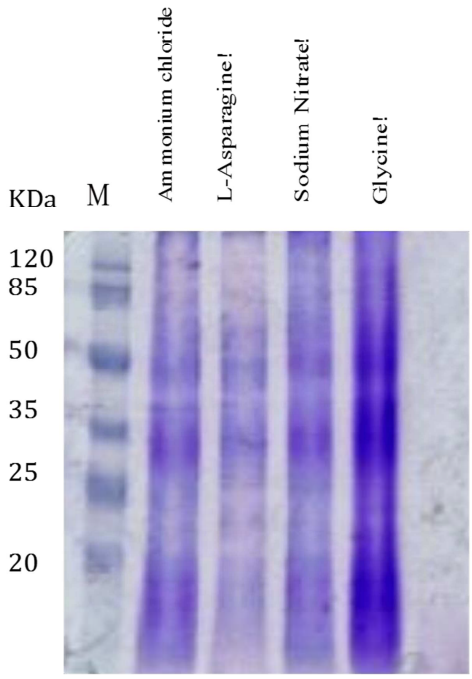


Figure 9. SDS-PAGE pattern of protein extracts of *S. albus*. Culture filtrate protein preparations were precipitated using 10% (w/v) Trichloroacetic acid (TCA), washed with cold acetone, and mixed with 2x sample buffer before separation. (M, protein markers).

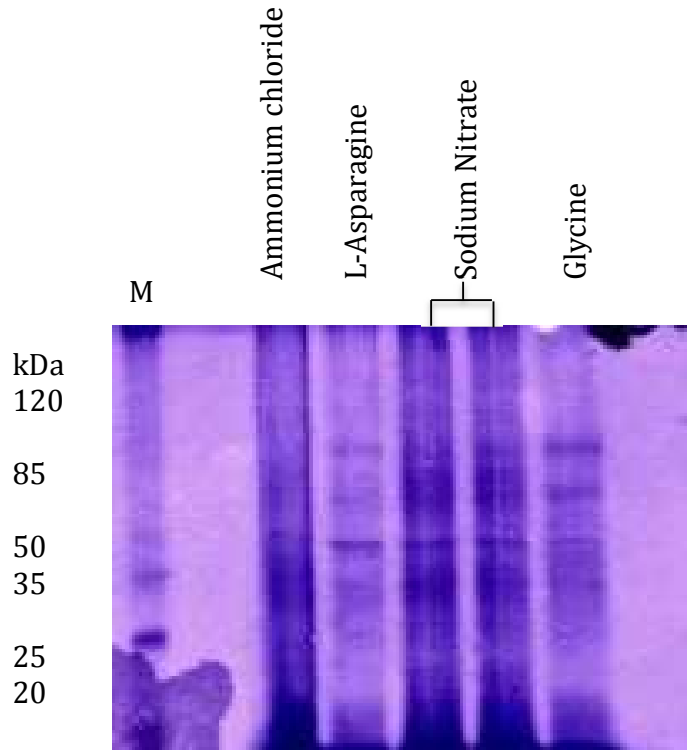


Figure 10. SDS-PAGE pattern of protein extracts of *S. albus*. Cytoplasmic protein preparations were precipitated using 10% (w/v) Trichloroacetic acid (TCA), washed with cold acetone, and mixed with 2x sample buffer before separation. (M, protein markers).

3.3.2 Phosphatase activity

The *API ZYM* system showed activity for both acid and alkaline phosphatases but since it is a qualitative test, a more quantitative test was needed to compare the effects of growth media composition and media pH. The activities of these enzymes were quantified by measuring the release of the yellow colored product *p*NP from the substrate *p*NPP. Having an established molar extinction coefficient it allows for the determination of specific enzyme activities. The highest specific activity of acid phosphatase was observed in the cytoplasmic protein fraction of the bacterium cultured in media with sodium nitrate as a nitrogen source (Figure 11). In the culture filtrate proteins fraction however the highest activity was observed in cultures utilizing glycine as a nitrogen source (Figure 12). The highest activity in the cytoplasmic fractions was 4-fold higher than that observed in the culture filtrate proteins fractions. Similarly, the highest specific activity of alkaline phosphatase was observed in the cytoplasmic protein fraction of the bacterium cultured in media with sodium nitrate as a nitrogen source (Figure 13). In contrast however, the culture filtrate proteins fraction with highest activity was observed in cultures utilizing L-asparagine as a nitrogen source (Figure 14). Here again the activity of the cytoplasmic fractions showed in general higher activity than those observed in the culture filtrate proteins (up to 6 fold higher).

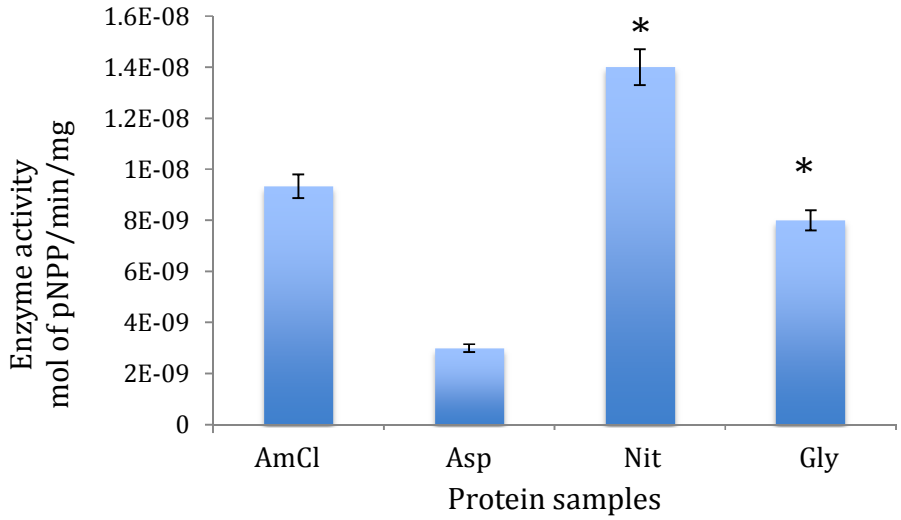


Figure 11. Acid phosphatase activity of *S.albus* cytoplasmic extracts. The bacterium was cultured in Sauton's media containing glucose as a carbon source and as a nitrogen source ammonium chloride (AmCl), L-asparagine (Asp), sodium nitrate (Nit), or glycine (Gly). Values are expressed as mean ± SEM. (*,*; $P < 0.05$). The highest specific activity of acid phosphatase was observed in the cytoplasmic protein fraction of the bacterium cultured in media with sodium nitrate as a nitrogen source.

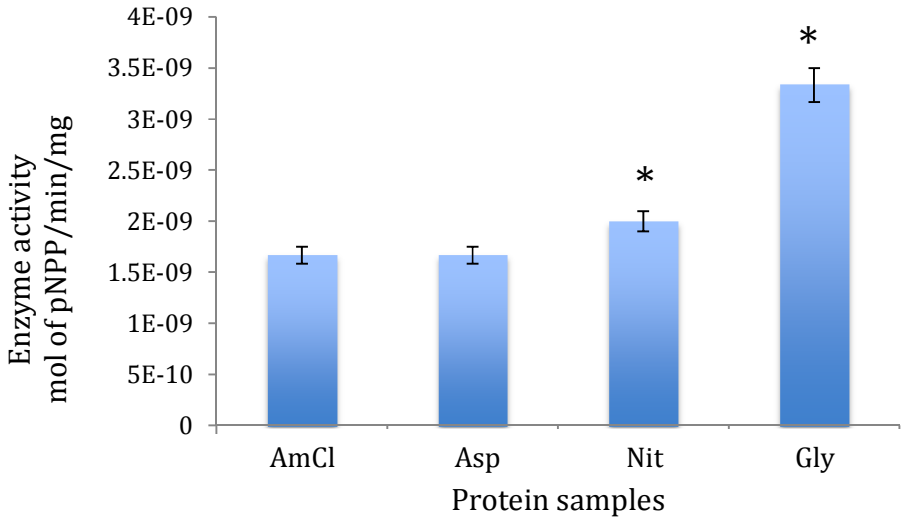


Figure 12. Acid phosphatase activity of *S.albus* culture filtrate proteins. The bacterium was cultured in Sauton's media containing glucose as a carbon source and as a nitrogen source ammonium chloride (AmCl), L-asparagine (Asp), sodium nitrate (Nit), or glycine (Gly). Values are expressed as mean ± SEM. (*,*; $P < 0.05$). The highest activity was observed in cultures utilizing glycine as a nitrogen source.

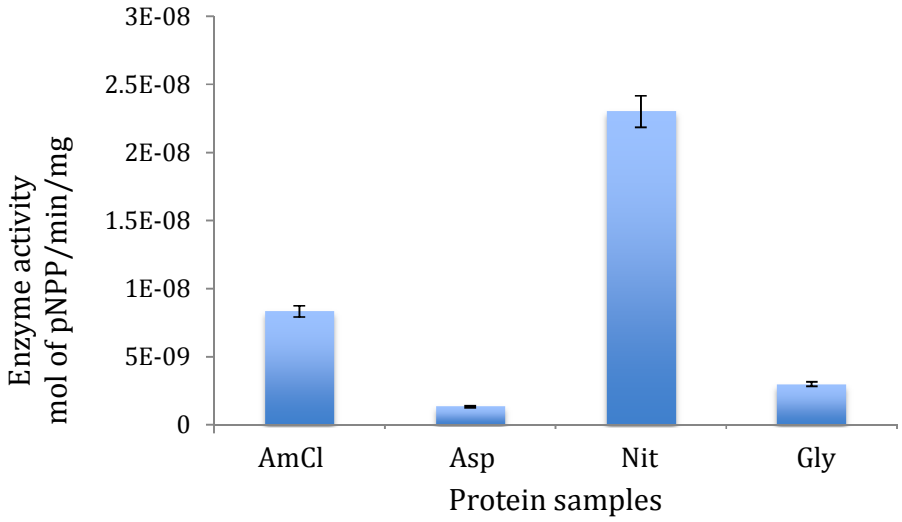


Figure 13. Alkaline phosphatase activity of *S.albus* cytoplasmic proteins. The bacterium was cultured in Sauton's media containing glucose as a carbon source and as a nitrogen source ammonium chloride (AmCl), L-asparagine (Asp), sodium nitrate (Nit), or glycine (Gly). Values are expressed as mean ± SEM. (*,*; $P < 0.05$). The highest specific activity was observed in the cytoplasmic protein fraction of the bacterium cultured in media with sodium nitrate as a nitrogen source.

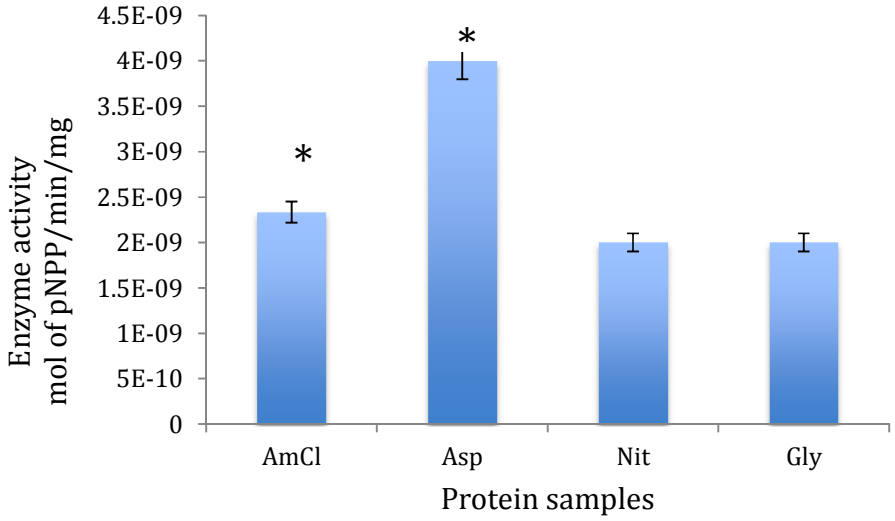


Figure 14. Alkaline phosphatase activity of *S.albus* culture filtrate proteins. The bacterium was cultured in Sauton's media containing glucose as a carbon source and as a nitrogen source ammonium chloride (AmCl), L-asparagine (Asp), sodium nitrate (Nit), or glycine (Gly). Values are expressed as mean ± SEM. (*,*; $P < 0.05$). The highest activity was observed in cultures utilizing L-asparagine as a nitrogen source.

3.3.3 Alanine dehydrogenase activity

As significant alanine dehydrogenase activity has been reported in the culture supernatant of *M. tuberculosis*, the activity of this enzyme was also investigated in the cultures of *S. albus*. Since this enzyme is considered a cytoplasmic enzyme it was not surprising to detect significant activity in the cytoplasmic fractions (Figure 15) with particularly high activity in the cytoplasmic fraction of the bacterium cultured in Sauton's media with glycine as a nitrogen source. What was surprising however is the equivalent activity detected in the culture filtrate proteins fractions from Sauton's media containing sodium nitrate as a nitrogen source (Figure 16)?

3.3.4 Alcohol dehydrogenase activity

The activity of this enzyme was followed by measuring the formation of NADH from the dehydrogenation of ethanol. The activity of this enzyme was negligible in both cytoplasmic and culture filtrate proteins fractions of the bacterium cultured in Sauton's medium with L- asparagine as a nitrogen source (Figure 17-18). Low activity was also observed in the cytoplasmic fractions from media with sodium nitrate as a nitrogen source (Figure 17). Curiously, the culture supernatant fraction of this medium showed the highest activity of all fractions tested.

3.3.5 Peroxidase activity

Peroxidase is a test not included in the *API ZYM* strips. Strong catalase activity was observe in both the cytoplasmic and culture filtrate proteins fractions of the bacterium cultured in Sauton's media with either sodium nitrate or glycine as a nitrogen

source (Table 7). Although activity was observed in all other fractions, the lowest activities were observed in the culture filtrate proteins fractions of the bacterium cultured in Sauton's media with L-asparagine and ammonium chloride as nitrogen sources.

3.3.6 Catalase activity

The *API ZYM* strips used earlier in the work do not include a test for catalase activity. Aerobic microorganisms typically produce this class of enzymes in addition to catalases to assist with detoxification of reactive oxygen. It was surprising to see a "disconnect" between these two activities (compare Table 7 to Figure 19-20). Amongst the cytoplasmic fractions significant activity was detected only in cultures of Sauton's media utilizing glycine as a nitrogen source (Figure 19). Equally surprising was the finding that the activity of this enzyme was negligible in the culture supernatants from cultures in Sauton's media with L- asparagine as a nitrogen source (Figure 20).

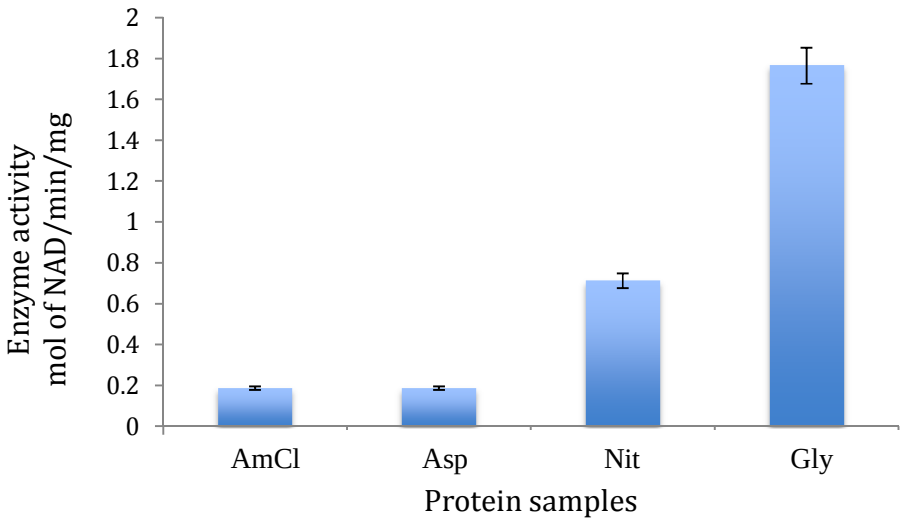


Figure 15. Alanine dehydrogenase levels in *S. albus* cytoplasmic. Values are expressed as mean ± SEM. (*, * and **, **; $P < 0.05$). High activity in the cytoplasmic fraction of the bacterium cultured in Sauton's media with glycine. AmCl (ammonium chloride), Asp (asparagine), Nit (nitrate), and Gly (glycine).

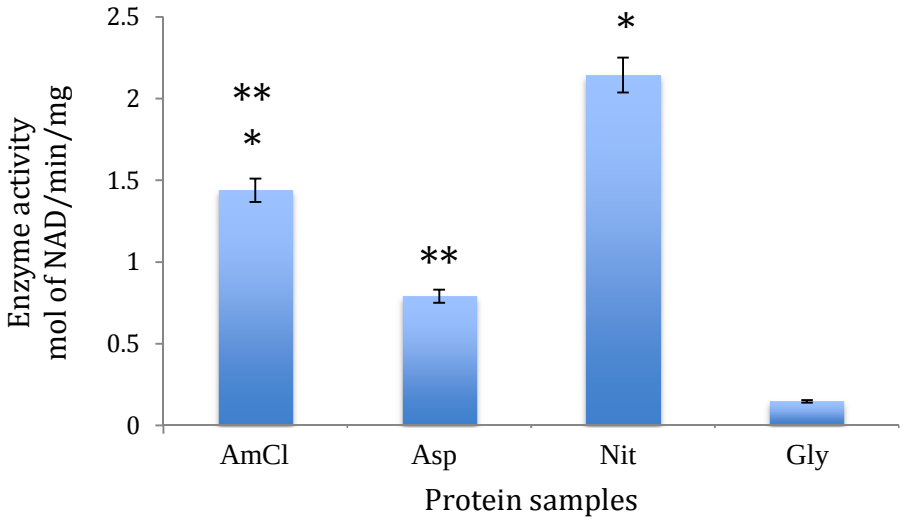


Figure 16. Alanine dehydrogenase levels in *S.albus* culture filtrate proteins fractions. Values are expressed as mean ± SEM. (*, * and **, **; $P < 0.05$). The equivalent activity detected in the culture filtrate proteins fractions from Sauton's media containing sodium nitrate as a nitrogen source. AmCl (ammonium chloride), Asp (asparagine), Nit (nitrate), and Gly (glycine).

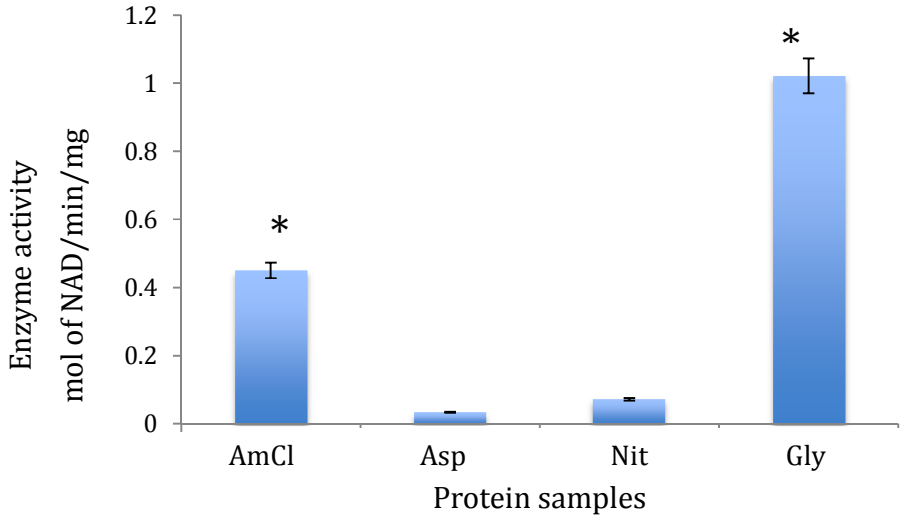


Figure 17. Alcohol dehydrogenase activity in cytoplasmic proteins of *S.albus.* Values are expressed as mean ± SEM. ($P < 0.05$). The activity of this enzyme was negligible in bacterium cultured in Sauton's medium with L-asparagine.

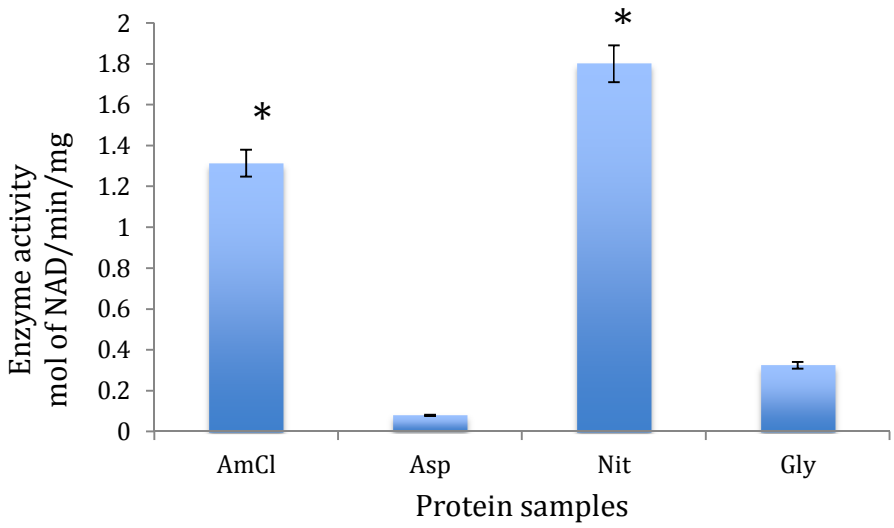


Figure 18. Alcohol dehydrogenase activity in culture filtrate proteins (b) of *S.albus*. Values are expressed as mean ± SEM. ($P < 0.05$). The activity of this enzyme was negligible in bacterium cultured in Sauton's medium with L-asparagine. AmCl (ammonium chloride), Asp (asparagine), Nit (nitrate), and Gly (glycine).

Table 7. Peroxidase activity in the culture filtrate proteins and cytoplasmic fractions of *S. albus* in different Sauton's medium. Strong catalase activity was observe in both the cytoplasmic and culture filtrate proteins fractions of the bacterium cultured in Sauton's media with either sodium nitrate or glycine as a nitrogen source. The number of marks (+) means increase of the activity.

Medium	Culture filtrate	Cytoplasmic
L-Asparagine	+	++
Ammonium chloride	+	++
Sodium nitrate	+++	+++
Glycine	+++	+++

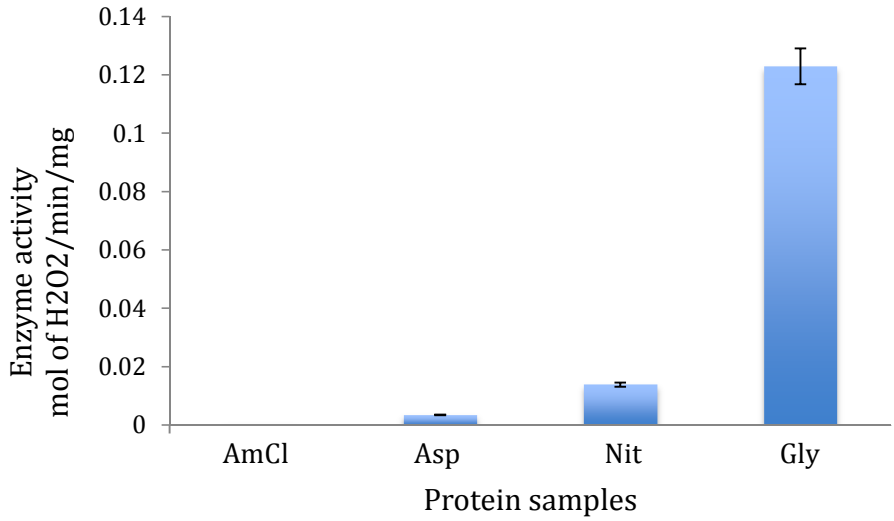


Figure 19. Catalase activity in cytoplasmic protein fractions of *S.albus*. Values are expressed as mean ± SEM. Significant activity was detected only in cultures of Sauton's media utilizing glycine. AmCl (ammonium chloride), Asp (asparagine), Nit (nitrate), and Gly (glycine).

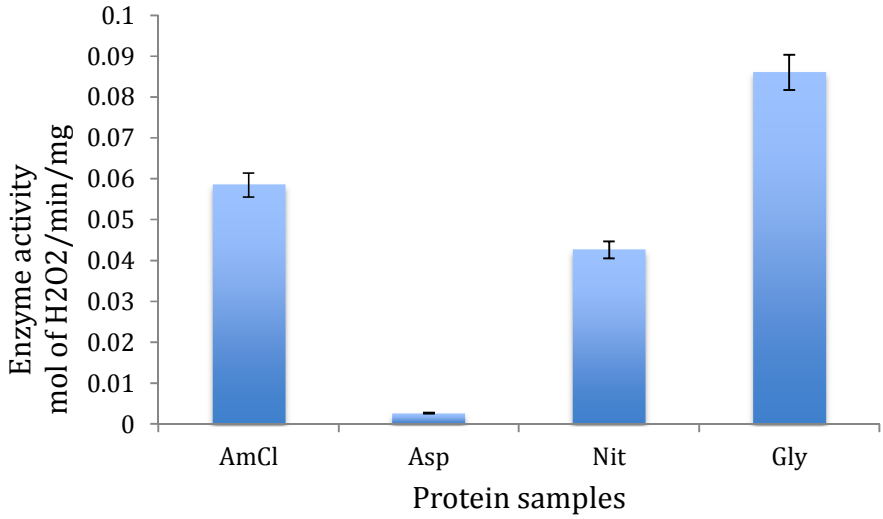


Figure 20. Catalase activity in cytoplasmic protein (A) and culture filtrate proteins (B) fractions of *S.albus*. Values are expressed as mean ± SEM. the activity of this enzyme was negligible in the culture supernatants from cultures in Sauton's media with L-asparagine.

3.3.7 Matrix Metalloproteinase/Gelatinase activity

Gelatin zymography is a powerful technique that is used to detect the ability of proteolytic enzymes to degrade gelatin, specifically the matrix metalloproteinase MMP-2 (gelatinase A) and MMP-9 (gelatinase B) type of enzymes (Toth and Fridman, 2001). Figure 20 shows the degradation of gelatin by streptomyces enzymes. Equivalent and strong activities were detected in the cytoplasmic fractions from media with ammonium chloride and sodium nitrate as nitrogen sources. Potentially five distinct bands can be resolved within the gel (Figure 21 A and B). Weaker activity with only two bands could be seen in lanes containing the cytoplasmic fraction of the media with glycine as a nitrogen source. These results were obtained regardless of whether $CaCl_2$ or $ZnCl_2$ were included in the incubation buffer. The bands correspond to the following approximate masses: 120 kDa, 50 kDa, 35 kDa, 23 kDa, and 18 kDa. The only activity detected in the culture supernatants was from media containing sodium nitrate as a nitrogen source (Figure 21 A) where a single band of approximately50 kDa in size was visible. This was reproducible in incubation buffer containing $CaCl_2$ but not $ZnCl_2$. Upon concentration of the supernatants and/or prolonged incubation times, more clear bands appear, particularly in the supernatant from the media with glycine as a nitrogen source. The gels were Zinc chloride in the incubation buffer (Figure 22). Two distance bands at approximately 50 kDa and 18 kDa can be recognized. The smaller of the two can be recognized in the other supernatants.

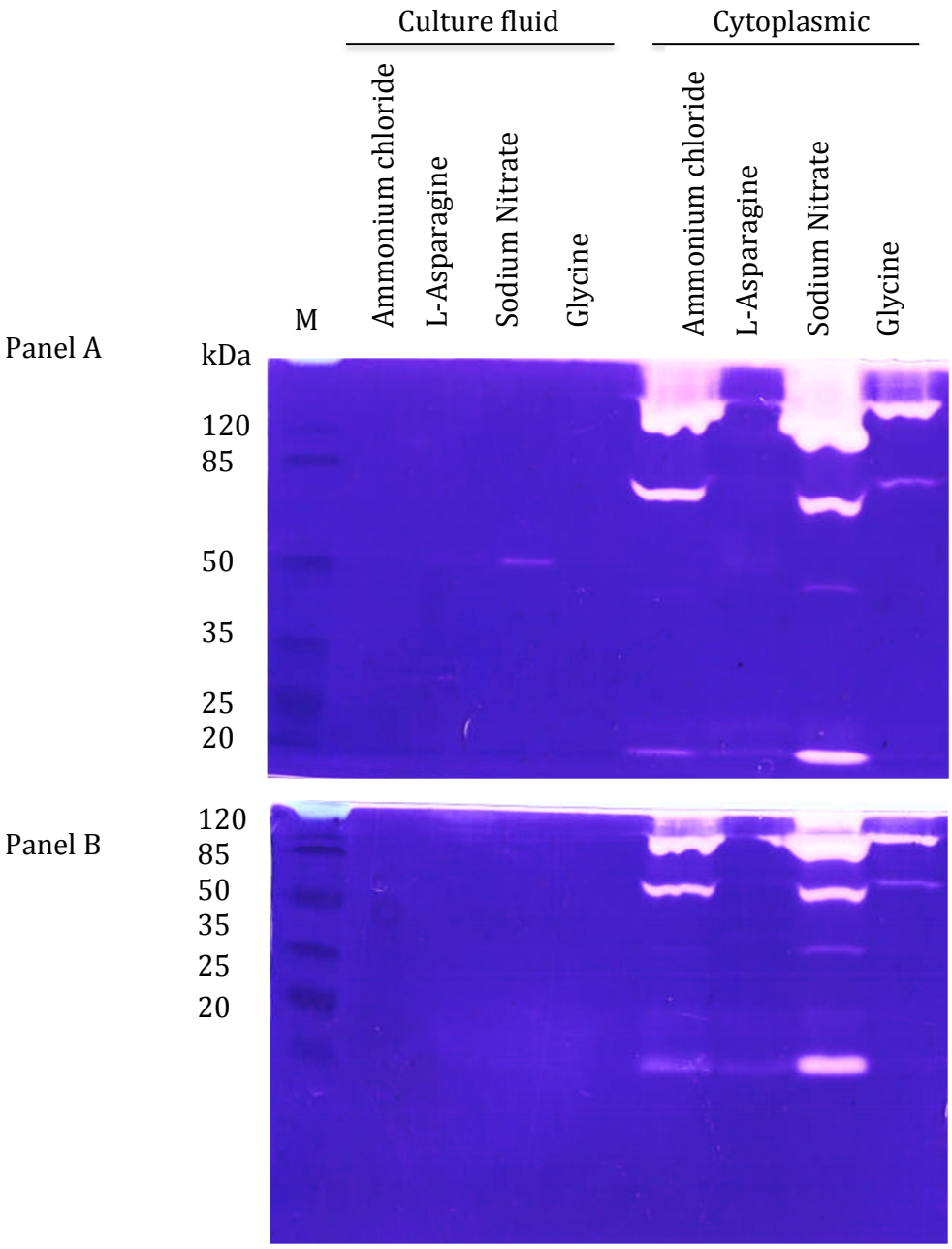


Figure 21. Gelatin zymogram of *S. albus* fractions. The gels were either developed with calcium chloride (A) or with Zinc chloride (B) in the incubation buffer. Potentially five distinct bands can be resolved within the gel in cytoplasmic fraction in both and weak degradation for the secreted protein in both.

3.4 Cross reactivity with Mycobacteria-specific antibodies

Because of the close phylogenetic relatedness of both mycobacteria and streptomyces and the fact both *M. tuberculosis* and this clinical isolate of *S. albus* are respiratory pathogens, it was anticipated that not only they may share secreted enzymatic profile but also protein homologies. At least one secreted enzyme of *M. tuberculosis*, SapM, shares significant sequence homology 55% identity and 71% positively relative .It was then decided to extend the enzymatic profiling and perform immunological profiling to assess the level of homologies in the secreted proteins of both pathogens. Nine different monoclonal antibodies were used for this screening. Two monoclonal antibodies generated in our laboratory against peptides from SapM and one against *M. tuberculosis* alanine dehydrogenase (AlaDH) whole protein (Table 8). Six additional monoclonal antibodies kindly provided to us by Colorado State University against other secreted antigens of *M. tuberculosis* as indicated in Table 8 below. Of the two anti-SapM antibodies used, F181-ID3-2 reacted with two bands of approximately 28 kDa and 21 kDa size in all four supernatants tested (Figure 23). None of the cytoplasmic extracts reacted with this antibody. When culture filtrate proteins of *M. bovis* BCG are used as a positive control, two bands become visible in the western blots developed with anti-AlaDH antibodies (Figure24).Also, using CFP ofH37Rv as a control, two visible bands in the western blot developed with (HspX, NR-13607) - Rv2031c HspX (α-crystallin). However, none of the *S. albus* extracts reacted with this antibody. All other monoclonal antibodies fail to react with any proteins in the cytoplasmic or culture supernatant fractions tested. The results are summarized in Table 8 below.

Table 8. Cross reactivity of different Mycobacteria-specific antibodies with protein extracts of this clinical isolate of *S. albus.*

Antibody	Type	Antigen	Result
ANTI-4287, F181-5F12-2		Rv3310 (SapM)	-
NR-13607		Rv2031c HspX (α-crystallin)	-
NR-13804		Rv0475 (iron-regulated heparin-binding hemagglutinin (HBHA))	-
ANTI-4287, F181-4C2-2		Rv3310 SapM	-
NR-13605		Rv0934 (periplasmic phosphate-binding lipoprotein)	-
NR-13817		Rv1860 (MPT32))	-
NR-13793		Rv1908c (catalase-peroxidase)	-
ANTI-ALADH, F181-5D6-2		ALAD	+
ANTI-4287, F181-ID3-2		Rv3310 SapM	+

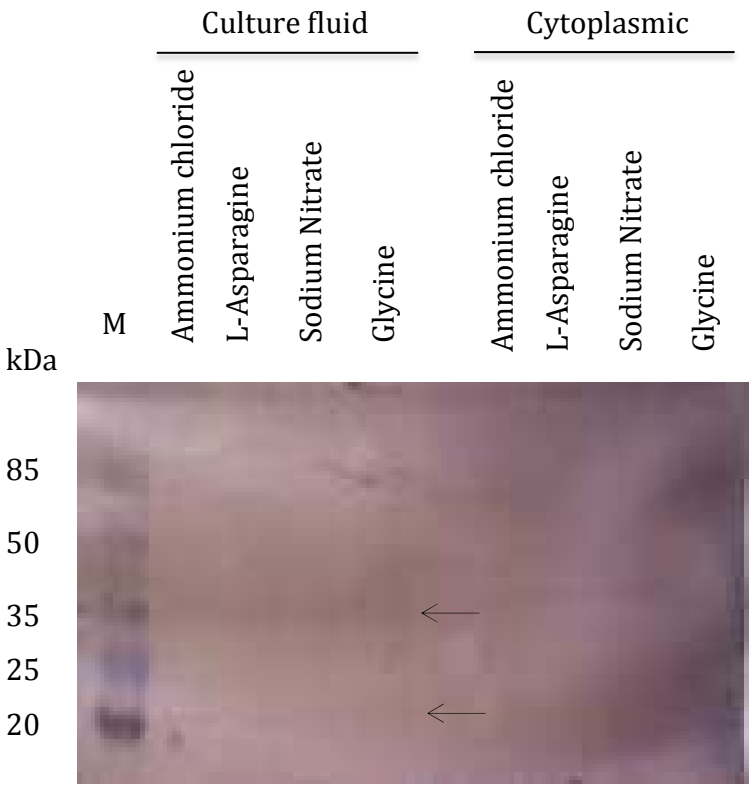


Figure 23. Cross reactivity of Mycobacteria-specific antibody (F181-ID3-2) with proteins from *S. albus*. Arrows indicate locations where positive reactivity was observed.

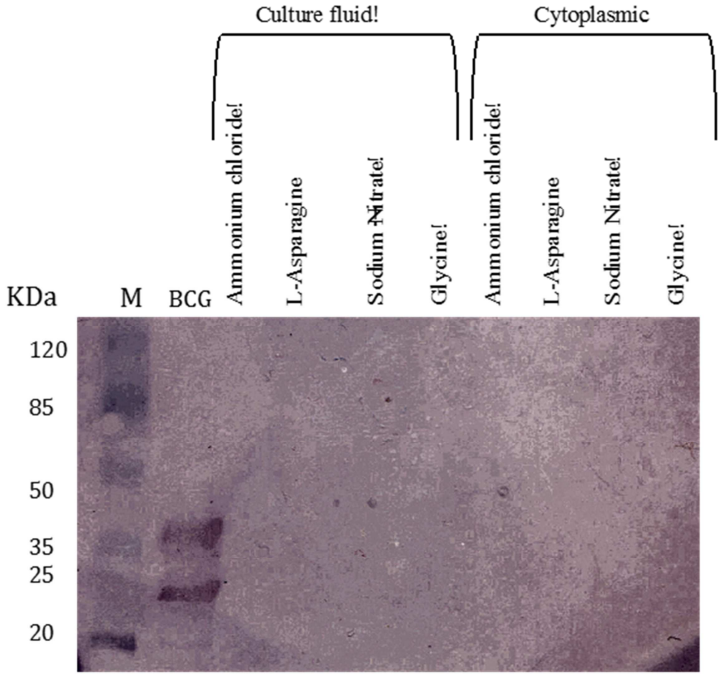


Figure 24. Cross reactivity of Mycobacteria-specific antibody (ANTI-AlaDH) with proteins from *S. albus*. BCG is used as a positive control. (M: protein markers).

4. Discussion

This study established a functional relationship between *S. albus*, which is known as an appropriate host for the heterologous production of bioactive secondary metabolites *(Zaburannyi et al., 2014)*, and *M. tuberculosis* by comparing their enzymatic and immunological profiles. Beginning with the interesting findings in the growth experiments of *S. albus* that was grown in a defined minimal medium (*M. tuberculosis* Sauton's media) originally developed for mycobacteria (Sassetti et al., 2003). This medium was selected in this study because it is well defined, minimal, and protein/peptide free so as to facilitate proteomic analysis in the extension of this study. The acidification of the growth medium in stationary phase when asparagine and ammonium chloride were used as a nitrogen source by the *S. albus*: a drop in pH from the initial pH of 7.4 to pH about 5.8. This drop in pH was also observed when *M. tuberculosis* and *M. bovis* BCG are cultured in Sauton's medium containing ammonium chloride as a nitrogen source. Although was not confirmed in this study, the most likely reason for these observations is the excretion and accumulation of organic acids during stationary and late phases of growth.

The profiles of enzyme activities in the four culture media tested (Sauton's medium with the nitrogen source being either ammonium chloride, asparagine, nitrate, or glycine) were similar but with notable differences (Tables 5 and 6). Enzymes activities that were detected only in media with glycine as a nitrogen source include β-galactosidase, β-glucosidase, and α-chymotrypsin. On the other hand, enzymes activities that were absent in this medium include β-glucuronidase and cysteine arylamidase. Another important

observation was that the profiles of enzymatic activities in the cytoplasmic fractions of the bacterium in the four culture media were different from those found in the secretory fractions, indicating that the latter are indeed secretory enzymes and not simply found in this fraction as a result of experimental error or cross contamination of the fractions during sample processing. These differences were confirmed by examining the protein banding pattern on SDS-PAGE (Figures 8 and 9). A previous study showed the activity of ten secreted enzymes by *M. tuberculosis* (Roman and Sicilia, 1984), eight enzymes activities out of those were also found in *S. albus* in this study. These enzyme activities were: alkaline phosphatase, esterase/lipases (C8), lipase (C14), leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, and β-glucosidase (Table 9).

Consistent with these findings, Raynaud et al. (1998) examined the extracellular enzyme activities in the culture supernatant of *M. tuberculosis*. Eight enzyme activities were detected only in the culture fluids and/or on the cell surface of this pathogen that are involved with the pathogenicity of this species. Some the enzyme activities detected in the present study with *S. albus* include alanine dehydrogenase, catalase, peroxidase, and alcohol dehydrogenase. Acid phosphates and alkaline phosphatase activities previously detected in *M. tuberculosis* and other species of non-pathogenic mycobacteria such as *M. smegmatis* were also detected in this study in *S. albus.* Importantly, this study showed that these enzyme activities were detected in both the cytoplasmic and the secretory fraction (spent culture medium).

Table 9. Comparison of 19 enzymes activities between *M. tuberculosis* (M) and *S. albus* (S), using the *API ZYM* test. Eight enzyme activities were found in both bacteria. (+) active (-) inactive.

	Enzyme	M	S
1	Alkaline phosphatase	+	+
2	Esterase (C 4)	+	-
3	Esterase Lipases (C 8)	+	+
4	Lipase (C 14)	+	+
5	Leucine arylamidase	+	+
6	Valine arylamidase	+	+
7	Cystine arylamidase	+	-
8	Trypsin	-	+
9	α-chymotrypsin	-	+
10	Acid phosphatase	+	+
11	Naphthol-AS-BI-phosphohydrolase	+	+
12	α-galactosidase	-	-
13	β-galactosidase	-	+
14	β-glucuronidase	-	-
15	α-glucosidase	-	-
16	β-glucosidase	+	+
17	N-acetyl-β-glucosaminidase	-	+
18	α-mannosidase	-	-
19	α-fucosidase	-	-

The levels of the following enzymes were further determined quantitatively: acid phosphatase, alkaline phosphatase, alanine dehydrogenase, alcohol dehydrogenase, and catalase/peroxidase (Table 10). Acid phosphatase was found in highest levels in the secretory fraction (CFP) of media containing glycine as a nitrogen source but was highest in the cytoplasmic fractions of cells grown with nitrate as a nitrogen source. This was interesting because this media did not show significant drop in pH as happened with media containing ammonium chloride and asparagine. This may indicate that this enzyme is not secreted in response to acid pH but is rather dependent on the nitrogen source directly. The highest activity of alkaline phosphatase was detected in the media with asparagine as a nitrogen source, also implicating its regulation in response to the nitrogen source directly and not the pH of the medium.

Nitrogen metabolism in streptomyces has been well defined. Nitrogen regulated genes (GlnR regulon) are under the control of the central repressor GlnR (Voelker and Altaba, 2001). This regulator controls the expression of three key enzyme systems involved in nitrogen assimilation: glutamine synthetase (GS), glutamate dehydrogenase (GDH), and glutamate synthase (GOGAT). These three enzymes assimilate inorganic nitrogen (ammonium ion or nitrate) into organic form through incorporation into glutamate and glutamine (Figure 25). Under high inorganic nitrogen conditions, as was used in this study, there will be accumulation of several organic acids such as 2-oxoglutarate and glutamate (Figure 25). It is therefore conceivable that the acidification of the media containing these nitrogen sources is due to excretion of excess 2-oxoglutarate and glutamate. In one study with *S. lividans*, excretion of 2-oxoglutarate as well as pyruvate was observed when the bacterium is cultured in media containing glucose as a carbon

Table 10. Summary of enzyme activity results that found mostly in the medium with glycine and sodium nitrate.

Enzyme	cytoplasmic	culture filtrate
Acid phosphatase	Sodium nitrate	Glycine
Alkaline phosphatase	Sodium nitrate	L-Asparagine
Peroxidase	Sodium nitrate + Glycine	Sodium nitrate + Glycine
Protease	Sodium nitrate	Sodium nitrate +Glycine
Alanine dehydrogenase	Glycine	Sodium nitrate
Alcohol dehydrogenase	Glycine	Sodium nitrate
Catalase	Glycine	Glycine

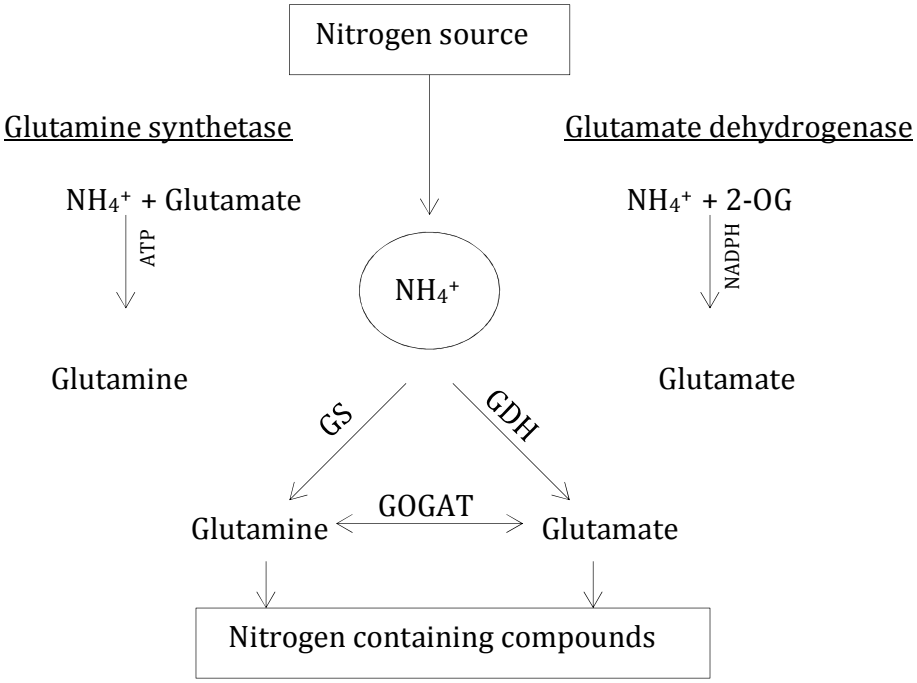


Figure 25. Control of nitrogen assimilation in streptomyces. Adapted from Voelker and Altaba (2001).

source and nitrate as a nitrogen source (Madden et al., 1996). In a similar study but with *S. peucetius*, the same organic acids were found to be excreted in culture media (Dekleva and Strohl, 1987). In this study, secretion of the acid phosphatase activity did not correlate with acidification of the media. Non-specific acid phosphatases (such as SapM) are typically regulated by inorganic phosphate levels rather than acid pH (Tiffert et al., 2008)

Alanine dehydrogenase, alcohol dehydrogenase, and catalase/peroxidase showed highest levels in the CFP fraction of the media supplemented with nitrate as the nitrogen whereas it was highest in the cytoplasmic fraction of cells harvested from media with glycine as the nitrogen source. These enzymes were investigated in more details because of their potential involvement in the virulence of this pathogen. Another type of enzyme activity often implicated in the virulence of intracellular pathogens is metalloproteinases. This activity was assessed in both the secretory fraction and the cytoplasmic fraction of *S. albus* grown in all four media. Gelatinase zymography showed that the cytoplasmic fractions of cells grown in Sauton's media with ammonium chloride and nitrate as nitrogen sources contained the highest activities. The zymograms showed two distinct major bands corresponding to approximately 120 kDa and 70 kDa and two minor bands at 48 kDa and 20 kDa (Figure 15). It is not known whether these bands correspond to four distinct proteins or to proteolytic fragments of a fewer number of proteins. In the secretory fraction, one minor band was visible only in the medium with nitrate as a nitrogen source, corresponding to approximately 50 kDa in size.

The functional relatedness of *S. albus* and *M. tuberculosis* must be extended beyond similarity of enzymatic profiles. An important functional category is the immunological profile. While enzymatic profiles provide information on the

similarities in metabolism and protein secretion (with potential functions in pathogenesis), immunological profiles provide information on the capacity to provide a similar immunological response, a feature important in selecting a heterologous vaccine candidate. In this study, seven monoclonal antibodies specific for seven distinct antigens were used to screen for cross reactivity with the secretory fractions of *S. albus*. Of the seven antibodies, only one (F181-ID3-2) gave a positive hit. This is a monoclonal antibody directed at a specific internal amino acid sequence in the secreted acid phosphatase of mycobacteria (SapM). This protein has a size of about 28 kDa and is implicated in the pathogenesis of *M. tuberculosis* (Saleh and Belisle, 2000; Vergne et al., 2005). All other antibodies were negative in their reactivity. One must keep in mind that these are monoclonal antibodies and are specific against small and well-defined epitopes and thus require a very high degree of homology between two different proteins from two different bacteria to give a positive reaction. Antibodies against Mycobacteria antigens also reacted with antigens from streptomyces, suggesting that not only do they share enzyme activities but also share protein sequences and has implications on relatedness of immunological responses induced by these related pathogens.

5. Conclusions

A significant aspect of the present work is the finding the quantities or qualitative evaluation of enzyme activity, of the 14 comparative enzymes (acid phosphatase, alkaline phosphatase, peroxidase, protease, alanine dehydrogenase, alcohol dehydrogenase, catalase, superoxide dismutase, esterase, lipases (C8), lipase (C14), leucine arylamidase, valine arylamidase, naphthol-AS-BI-phosphohydrolase, and β–glucosidase) are found in both *S. albus* and *M. tuberculosis*. This finding suggests that streptomyces and mycobacteria share similar growth and metabolic properties. On the other hand, the fact that some antibodies against mycobacteria antigens also react with antigens from streptomyces also suggests that not only do they share enzyme activities but also share protein sequences and has implications on relatedness of immunological responses induced by these related pathogens.

This enzymatic study confirms that both species are not only phylogenetically but also physiologically related. It also shows that these enzymatic profiles are dependent on the growth media used to culture the bacteria. This could be important in selecting the appropriate streptomyces species and in formulating the growth media for a vaccine candidate for TB.

The next objective will be to further investigate the immunological cross reactivity of these two actinobacteria and study the immune response to *S. albus* as a new live vaccine vehicle strategy against TB. An animal-based study whereby a genetically engineered *S. albus* or a related specie expressing specific antigens of *M. tuberculosis* is used for immunization and the animals are then challenged with an infectious dose of *M. tuberculosis* would be an appropriate line of investigation to follow.

Bibliography

Adelantado, C., Shiva, C., Arosemena, L., Costa-Batllori, P., and Calvo, M. A. (2007). Enzymatic mechanisms related to antimicrobial activity of rutaceae extracts. *J. Biol. Sci*., **7**:71-73.

Anderson, A. S., and Wellington, E. M. (2001). The taxonomy of streptomyces and related genera. *Int. J. Syst. Evol. Microbiol*., **51**:797-814.

Andersen, P. (2001). TB vaccines: progress and problems. *Trends Immunol*., **22**:160-168.

Atkinson, J. J., and Senior, R. M. (2003). Matrix metalloproteinase-9 in lung remodeling. *Am. J. Respir. Cell Mol. Biol.*, **28**:12-24.

Arzuaga, N. O., Granda, A. V., Gómez, J. C. R., San Miguel, M. E. S. G., Bourzac, J. F. I., Hernández, Y. L., ... and Domínguez, A. A. (2011). The use of streptomyces for immunization against mycobacterial infections. *Hum. Vaccines* **7**:934-940.

Avasthi, R., Chaudhary, S. C., and Mohanty, D. (2009). Giant mantoux reaction. *Indian J. Med. Microbiol*., **27**:78-79.

Basak, K., and Majumdar, S. K. (1973). Utilization of carbon and nitrogen sources by *Streptomyces kanamyceticus* for kanamycin production. *Antimicrob. Agents Chemother.,* **4**:6-10.

Beers, R. F., and Sizer, I. W. (1952). A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J. Biol. Chem.,* **195**:133-140.

Behr, M. A. (2002). BCG - different strains, different vaccines? *Lancet Infect. Dis.,* **2**:86-92.

Binnie, C., Douglas Cossar, J., and Stewart, D. I. (1997). Heterologous biopharmaceutical protein expression in streptomyces. *Trends Biotechnol.*, **15**:315-320.

Blackwood, K. S., He, C., Gunton, J., Turenne, C. Y., Wolfe, J., and Kabani, A. M. (2000). Evaluation of recA sequences for identification of mycobacterium species. *J. Clin. Microbiol*, **38**:2846-2852.

Blandino, A., Caro, I., and Cantero, D. (1997). Comparative study of alcohol dehydrogenase activity in flor yeast extracts. *Biotechnol. Lett.,* **19**:651-654.

Brust, B., Lecoufle, M., Tuaillon, E., Dedieu, L., Canaan, S., Valverde, V., and Kremer, L. (2011). Mycobacterium tuberculosis lipolytic enzymes as potential biomarkers for the diagnosis of active tuberculosis. *PloS one*, **6**: e25078.

Castillo-Rodal, A. I., Castañón-Arreola, M., Hernández-Pando, R., Calva, J. J., Sada-Díaz, E., and López-Vidal, Y. (2006). *Mycobacterium bovis* BCG substrains confer different levels of protection against *Mycobacterium tuberculosis* infection in a BALB/c model of progressive pulmonary tuberculosis. *Infect. Immun.*, **74**:1718-1724.

Ceylan, O., Okmen, G., and Ugur, A. (2008). Isolation of soil Streptomyces as source antibiotics active against antibiotic-resistant bacteria. *J. Biosci*., **2**:73-82.

Chakrabarti, S., and Patel, K. D. (2005). Matrix metalloproteinase-2 (MMP-2) and MMP-9 in pulmonary pathology. *Exp. Lung. Res.*, **31**:599-621.

Chapman, A. L., Munkanta, M., Wilkinson, K. A., Pathan, A. A., Ewer, K., Ayles, H., and Lalvani, A. (2002). Rapid detection of active and latent tuberculosis infection in HIV-positive individuals by enumeration of *Mycobacterium tuberculosis*-specific T cells. *Aids*, **16**:2285-2293.

Chen, J. M., Alexander, D. C., Behr, M. A., and Liu, J. (2003). *Mycobacterium bovis* BCG vaccines exhibit defects in alanine and serine catabolism. *Infect. Immun.*, **71**:708-716.

Cole, S. T. (2003). Recombinant BCG exporting ESAT-6 confers enhanced protection against tuberculosis. *Nature Med.*, **9**:533-539.

Corbett, E. L., Watt, C. J., Walker, N., Maher, D., Williams, B. G., Raviglione, M. C., and Dye, C. (2003). The growing burden of tuberculosis: global trends and interactions with the HIV epidemic. *Arch. Intern. Med.*, **163**:1009-1021.

Daniel, T. M. (2006). The history of tuberculosis. *Respir. Med.*, **100**:1862-1870.

Datta, P., Arora, S., Jain, R., Chander, J., and van de Sande, W. (2012). Secondary Peritonitis Caused by *Streptomyces viridis*. *J. Clin. Microbiol*., **50**:1813-1814.

Dekleva, M. L. and William R. Strohl (1987) Glucose-stimulated acidogenesis by *Streptomyces peucetius*. *Can. J. Microbiol*., 1987, **33**:1129-1132.

Devulder, G., De Montclos, M. P., and Flandrois, J. P. (2005). A multigene approach to phylogenetic analysis using the genus mycobacteria as a model. *Int. J. Syst. Evol. Microbiol.*, **55**:293-302.

Embley, T. M., and Stackebrandt, E. (1994). The molecular phylogency and systematics of the actinomycetes. *Annu. Rev. Microbiol*., **48**:257-289.

Fahal, A. H. (2004). Mycetoma: a thorn in the flesh. *Trans. R. Soc. Trop. Med. Hyg*., **98:**3-11.

Fauci, A. S., Touchette, N. A., and Folkers, G. K. (2005). Emerging infectious diseases: a 10-year perspective from the National Institute of Allergy and Infectious Diseases. *Int. J. Risk Saf. Med*., **17**:157-167.

Flärdh, K., and Buttner, M. J. (2009). Streptomyces morphogenetics: dissecting differentiation in a filamentous bacterium. *Nat. Rev. Microbiol.*, **7**:36-49.

Flynn, J. L. (2004). Immunology of tuberculosis and implications in vaccine development. *Tubercle*, **84**:93-101.

Garcia-Gonzalez, M. D., Martin, J. F., Vigal, T. O. M. A. S., and Liras, P. A. L. O. M. A. (1991). Characterization, expression in *Streptomyces lividans*, and processing of the amylase of *Streptomyces griseus* IMRU 3570: two different amylases are derived from the same gene by an intracellular processing mechanism. *J. Bacteriol*., **173**:2451-2458.

Gengenbacher, M., and Kaufmann, S. H. (2012). *Mycobacterium tuberculosis*: success through dormancy. *FEMS Microbiol. Rev*., **36**:514-532.

Ginsberg, A. M. (2002). What's new in tuberculosis vaccines? *Bull.WHO,* **80**:483-488.

Ginsberg, A. M., and Spigelman, M. (2007). Challenges in tuberculosis drug research and development. *Nature*, **13**:290-294.

Gouzy, A., Larrouy-Maumus, G., Bottai, D., Levillain, F., Dumas, A., Wallach, J. B., and Neyrolles, O. (2014). *Mycobacterium tuberculosis* Exploits Asparagine to Assimilate Nitrogen and Resist Acid Stress during Infection. *PLoS pathog.*, **10**: e1003928.

Gutierrez, M. C., Brisse, S., Brosch, R., Fabre, M., Omaïs, B., Marmiesse, M., and Vincent, V. (2005). Ancient origin and gene mosaicism of the progenitor of *Mycobacterium tuberculosis*. *PLoS Pathog.*, **1**: e5.

Han, J. S., Cheng, J. H., Yoon, T. M., Song, J., Rajkarnikar, A., Kim, W. G., Yoo, I. D., Yang, Y. Y. and Suh, J. W. (2005). Biological control agent of common scab disease by antagonistic strain *Bacillus sp*. sunhua. *J. Appl. Microbiol.*, **99**:213-221.

Hanekom, W. A. (2010). MVA85A, a novel TB vaccine, is safe in adolescents and children, and induces complex subsets of polyfunctional CD4+ T cells. *Eur. J. Immunol.*, **40**:279.

Hatzios, S. K., and Bertozzi, C. R. (2011). The regulation of sulfur metabolism in *Mycobacterium tuberculosis. PLoS Pathog.*, **7**: e1002036.

Hedges, S. B. (2002). The origin and evolution of model organisms. *Nat. Rev. Genet*., **3**:838-849.

Hingley-Wilson, S. M., Sambandamurthy, V. K., and Jacobs, W. R. (2003). Survival perspectives from the world's most successful pathogen, *Mycobacterium tuberculosis. Nat. Immunol.*, **4**:949-955.

Hoffmann, C., Leis, A., Niederweis, M., Plitzko, J. M., and Engelhardt, H. (2008). Disclosure of the mycobacterial outer membrane: cryo-electron tomography and vitreous sections reveal the lipid bilayer structure. *Proc. Natl. Acad. Sci.* (USA), **105**:3963-3967.

Hutter, B., and Dick, T. (1998). Increased alanine dehydrogenase activity during dormancy in *Mycobacterium smegmatis*. *FEMS Microbiol. Lett.,***167**:7-11.

Janda, J. M., and Abbott, S. L. (2007). 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *J. Clin. Microbiol.*, **45**:2761-2764.

Jensen, P. R., Williams, P. G., Oh, D. C., Zeigler, L., and Fenical, W. (2007). Species-specific secondary metabolite production in marine actinomycetes of the genus salinispora. *Appl. Environ. Microbiol.*, **73**:1146-1152.

Johnson, R., Streicher, E. M., Louw, G. E., Warren, R. M., Van Helden, P. D., and Victor, T. C. (2006). Drug resistance in *M. tuberculosis*. *Curr. Issues Mol. Biol.*, **8**:97-111.

Kagen, S. L., Fink, J. N., Schlueter, D. P., Kurup, V. P., and Fruchtman, R. B. (1981). *Streptomyces albus*: a new cause of hypersensitivity pneumonitis. *J. Allergy Clin. Immunol.*, **68**:295-299.

Kapadia, M., Rolston, K. V., and Han, X. Y. (2007). Invasive streptomyces infections six cases and literature review. *Am. J. Pathol.*, **127**:619-624.

Kaufmann, S. H. (2002). Protection against tuberculosis: cytokines, T cells, and macrophages. *Ann. Rheum. Dis.*, **61**(suppl 2): ii54-ii58.

Kaufmann, S. H. (2001). How can immunology contribute to the control of tuberculosis?. *Nat. Rev. Immunol.*, **1**:20-30.

Kaufmann, S. H. (2000). Is the development of a new tuberculosis vaccine possible?. *Nature Med.*, **6**:955-960.

Kaul, D. (2008). Coronin□1A epigenomics governs mycobacterial persistence in tuberculosis. *FEMS Microbiol. Lett.*, **278**:10-14.

Khaliq, S., Ghauri, M. A., and Akhtar, K. (2013). Isolation, identification and optimization of fermentation parameters for improved production of antimicrobial compounds from indigenous streptomyces isolates. *Afr. J. Microbiol. Res.*, **7**:1874-1887.

Kieser, T., Moss, M. T., Dale, J. W., and Hopwood, D. A. (1986). Cloning and expression of *Mycobacterium bovis* BCG DNA in" *Streptomyces lividans*". *J . bacteriol.*, **168**:72-80.

Kim, B. J., Lee, S. H., Lyu, M. A., Kim, S. J., Bai, G. H., Kim, S. J., ... and Kook, Y. H. (1999). Identification of mycobacterial species by comparative sequence analysis of the RNA polymerase gene (rpoB). *J. Clin. Microbiol.*, **37**:1714-1720.

Kirby, R., Sangal, V., Tucker, N. P., Zakrzewska-Czerwińska, J., Wierzbicka, K., Herron, P. R., ... and Hoskisson, P. A. (2012). Draft genome sequence of the human pathogen *Streptomyces somaliensis*, a significant cause of actinomycetoma. *J. bacterial.*, **194**:3544-3545.

Kleiner, D. E., and Stetlerstevenson, W. G. (1994). Quantitative zymography: detection of picogram quantities of gelatinases. *Anal. Biochem.*, **218**:325-329.

Kontro, M., Lignell, U., Hirvonen, M. R., and Nevalainen, A. (2005). pH effects on 10 *Streptomyces spp.* growth and sporulation depend on nutrients. *Lett. Appl. Microbiol.*, **41**:32-38.

Kupeli, E., Karnak, D., Sak, S. D., and Kayacan, O. (2010). Hazards of the 'hard cash': hypersensitivity pneumonitis. *Can. Respir. J.*, **17**: e102.

Lichon, V., and Khachemoune, A. (2006). Mycetoma. *Am. J. Dermatopathol.*, **7**: 315-321.

Liu, J., Tran, V., Leung, A., Alexander, D., and Zhu, B. (2009). BCG vaccines. *Hum. vaccines*, **5**:70-8.

Lodders, N., and Kämpfer, P. (2007). Streptomycetaceae: Phylogeny, Ecology and Pathogenicity. *eLS pp. 2001*. DOI: 10.1002/ 9780470015902. a0020392.

Loria, R., Kers, J., and Joshi, M. (2006). Evolution of plant pathogenicity in streptomyces. *Annu. Rev. Phytopathol.*, **44**:469-487.

Madden, T., Ward, J. M., and Ison, A. P. (1996) Organic acid excretion by *Streptomyces lividans* TK24 during growth on defined carbon and nitrogen sources. *Microbiol*., **142**:3181-3185

Mahairas, G. G., Sabo, P. J., Hickey, M. J., Singh, D. C., and Stover, C. K. (1996). Molecular analysis of genetic differences between *Mycobacterium bovis* BCG and virulent M. bovis. *J. bacteriol.*, **178**:1274-1282.

McGuire, A. M., Weiner, B., Park, S. T., Wapinski, I., Raman, S., Dolganov, G., and Galagan, J. (2012). Comparative analysis of mycobacterium and related actinomycetes yields insight into the evolution of *Mycobacterium tuberculosis* pathogenesis. *BMC Genomics*, **13**(1), 120-147.

McKinney, J. D., zu Bentrup, K. H., Muñoz-Elías, E. J., Miczak, A., Chen, B., Chan, W. T., ... and Russell, D. G. (2000). Persistence of *Mycobacterium tuberculosis* in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. *Nature*, **406**:735-738.

McShane, H., Pathan, A. A., Sander, C. R., Goonetilleke, N. P., Fletcher, H. A., and Hill, A. V. (2005). Boosting BCG with MVA85A: the first candidate subunit vaccine for tuberculosis in clinical trials. *Tubercle*, **85**:47-52.

Moreno, C., Taverne, J., Mehlert, A., Bate, C. A., Brealey, R. J., Meager, A., and Playfair, J. H. (1989). Lipoarabinomannan from *Mycobacterium tuberculosis* induces the production of tumour necrosis factor from human and murine macrophages. *Clin. Exp. Immunol.*, **76**:240.

Ohashima, T. and Soda, K. (1979). Purification and properties of alanine dehydrogenase from *Bacillus sphaericus*. *Eur. J. Biochem.*, **100**:29-39.

Oettinger, T., Jørgensen, M., Ladefoged, A., Hasløv, K., and Andersen, P. (1999). Development of the *Mycobacterium bovis* BCG vaccine: review of the historical and biochemical evidence for a genealogical tree. *Tuber. Lung Dis.*, **79**:243-250.

Orme, I. M. (2001). The search for new vaccines against tuberculosis. *J. Leukoc. Biol.*, **70**:1-10.

Osborn, T. W. (1983). Changes in BCG strains. *Tubercle*, **64**:1-13.

Ottenhoff, T. H. and Kaufmann, S. H. (2012). Vaccines against tuberculosis: where are we and where do we need to go? *PLoS Pathog.*, **8**: e1002607. doi: 10.1371/journal.ppat.1002607

Pradel, E. and Boquet, P. L. (1988). Acid phosphatases of *Escherichia coli*: molecular cloning and analysis of agp, the structural gene for a periplasmic acid glucose phosphatase. *J. bacteriol.*, **170**:4916-4923.

Pym, A. S., Brodin, P., Majlessi, L., Brosch, R., Demangel, C., Williams, A., and Rashamuse, K., Magomani, V., Ronneburg, T., and Brady, D. (2009). A novel family VIII carboxylesterase derived from a leachate metagenome library exhibits promiscuous β-lactamase activity on nitrocefin. *Appl. Microbiol. Biotechnol.*, **83**:491-500.

Raviglione, M. C., Snider Jr, D. E., and Kochi, A. (1995). Global epidemiology of tuberculosis. *J. Amer. Med. Assoc. (JAMA)*, **273**:220-226.

Raynaud, C., Etienne, G., Peyron, P., Lanéelle, M. A., and Daffé, M. (1998). Extracellular enzyme activities potentially involved in the pathogenicity of *Mycobacterium tuberculosis*. *Microbiology*, **144**:577-587.

Reece, S. T. and Kaufmann, S. H. (2008). Rational design of vaccines against tuberculosis directed by basic immunology. *Int. J. Med. Microbiol.*, **298**:143-150.

Roman, M. C. and Sicilia, M. J. (1984). Preliminary investigation of *Mycobacterium tuberculosis* biovars. *J. Clin. Microbiol.*, **20**:1015-1016.

Saczko, J., Daczewska, M., Malarska1, A., Seweryn1, E., Saczko3, Z., Ogorzaeek4, A., and Banas, T. (2002). Catalase (CT) activity in placentas Complicated with pregnancy induced hypertension (PIH). *Zoologica Poloniae,* **47**:69-73.

Saleh, M. T. and Belisle, J. T. (2000). Secretion of an acid phosphatase (SapM) by Mycobacterium tuberculosis that is similar to eukaryotic acid phosphatases. *J. bacteriol.*, **182**:6850-6853.

Sassetti, C. M., Boyd, D. H., and Rubin, E. J. (2003). Genes required for mycobacterial growth defined by high density mutagenesis. *Mol. Microbiol.*, **48**:77-84.

Schaerlaekens, K., Van Mellaert, L., Lammertyn, E., Geukens, N., and Anné, J. (2004). The importance of the Tat-dependent protein secretion pathway in streptomyces as revealed by phenotypic changes in tat deletion mutants and genome analysis. *Microbiology*, **150**:21-31.

Scherr, N., and Nguyen, L. (2009). Mycobacterium versus Streptomyces - we are different, we are the same. *Curr. Opin. Microbiol.*, **12**:699-707.

Shleeva, M., Mukamolova, G. V., Young, M., Williams, H. D., and Kaprelyants, A. S. (2004). Formation of 'non-culturable'cells of *Mycobacterium smegmatis* in stationary phase in response to growth under suboptimal conditions and their Rpf-mediated resuscitation. *Microbiology*, **150**:1687-1697.

Schluger, N. W. and Rom, W. N. (1998). The host immune response to tuberculosis. *Am . J . Respir. Crit. Care Med.*, **157**:679-691.

Scriba, T. J., Tameris, M., Mansoor, N., Smit, E., van der Merwe, L., Isaacs, F., and Subramani, R., and Aalbersberg, W. (2012). Marine actinomycetes: An ongoing source of novel bioactive metabolites. *Microbiol. Res.* **167**:571-580.

Taylor, J. L., Hattle, J. M., Dreitz, S. A., Troudt, J. M., Izzo, L. S., Basaraba, R. J., and Izzo, A. A. (2006). Role for matrix metalloproteinase 9 in granuloma formation during pulmonary *Mycobacterium tuberculosis* infection. *Infect. Immun.*, **74**:6135-6144.

Tameris, M. D., Hatherill, M., Landry, B. S., Scriba, T. J., Snowden, M. A., Lockhart, S., and McShane, H. (2013). Safety and efficacy of MVA85A, a new tuberculosis vaccine, in infants previously vaccinated with BCG: a randomised, placebo-controlled phase 2b trial. *Lancet Infect. Dis* **381**:1021-1028.

Tieleman, L. N., Van Wezel, G. P., Bibb, M. J., and Kraal, B. (1997). Growth phase-dependent transcription of the *Streptomyces ramocissimus* tuf1 gene occurs from two promoters. *J. Bacteriol.*, **179**:3619-3624.

Tiffert, Y., Supra, P., Wurm, R., Wohlleben, W., Wagner, R., and J. Reuther (2008) The *Streptomyces coelicolor* GlnR regulon: identification of new GlnR targets and evidence for a central role of GlnR in nitrogen metabolism in actinomycetes. *Mol. Microbiol*., **67**:861-80.

Toossi, Z., Mayanja□Kizza, H., Hirsch, C. S., Edmonds, K. L., Spahlinger, T., Hom, D. L., and Whalen, C. W. (2001). Impact of tuberculosis (TB) on HIV□1 activity in dually infected patients. *Clin. Exp. Immunol.*, **123**:233-238.

Toth, M., and Fridman, R. (2001). Assessment of gelatinases (MMP-2 and MMP-9 by gelatin zymography. In *Metastasis research protocols* (pp. 163-174). Humana Press.

Vallin, C., Ayala, J. C., García-Rivera, D., Jones, J., Rodríguez, C., González, L., and Van Mellaert, L. (2009). Immune response to *Streptomyces lividans* in mice: A potential vaccine vehicle against TB. *Open Vaccine J.*, **2**: 85-91.

Ventura, M., Canchaya, C., Tauch, A., Chandra, G., Fitzgerald, G. F., Chater, K. F., and van Sinderen, D. (2007). Genomics of Actinobacteria: tracing the evolutionary history of an ancient phylum. *Microbiol. Mol. Biol. Rev.*, **71**:495-548.

Vergne, I., Chua, J., Lee, H. H., Lucas, M., Belisle, J., and Deretic, V. (2005). Mechanism of phagolysosome biogenesis block by viable *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci.* (USA), **102**:4033-4038.

Verver, S. 1., Warren, R. M., Beyers, N., Richardson, M., van der Spuy, G. D., Borgdorff, M. W., Enarson, D. A., Behr, M. A., and van Helden, P. D. (2005). Rate of reinfection tuberculosis after successful treatment is higher than rate of new tuberculosis. *Am. J. Respir. Crit. Care Med.*, **171**:1430-1435.

Verver, S., Warren, R. M., Beyers, N., Richardson, M., van der Spuy, G. D., Voelker, F., and Altaba, S. (2001). Nitrogen source governs the patterns of growth and pristinamycin production in '*Streptomyces pristinaespiralis*'. Microbiology, **147**:2447-2459.

WHO. World Health Organization (2014). Antimicrobial resistance: global report on surveillance 2014. *Bull.WHO.*

World Health Organization (2013).Global tuberculosis. *Bull.WHO.*

World Health Organization (2011). Recommendations to Assure the Quality, Safety and Efficacy of BCG Vaccines. *Bull.WHO*.

World Health Organization (2005). New Tuberculosis Therapy Offers Potential Shorter Treatment. *Bull.WHO*.

Zaburannyi, N., Rabyk, M., Ostash, B., Fedorenko, V., and Luzhetskyy, A. (2014). Insights into naturally minimized *Streptomyces albus* J1074 genome. *BMC genomics*, **15**:97-108.